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13. ABSTRACT (Maximum 200 Words)

During pregnancy the mammary epithelium and its supporting vasculature rapidly expand to prepare for lactation. To investigate the role of oxygenation and metabolism in these processes the oxygen-responsive component of the hypoxia-inducible factor (HIF)-1 complex, *HIF-1α*, was deleted in the murine mammary gland using the Cre/*lox*P system. Although vascular density remained similar, loss of *HIF-1α* impaired mammary differentiation and lipid metabolism, culminating in lactation failure and changes in milk composition (Objectives #1). Next, we investigated the effects of deletion of the *von Hipple lindau (VHL)* gene in the mammary gland, in order to stimulate constitutive over-expression of HIF-1α in the mammary epithelium, to determine if HIF-1α contributes to mammary gland tumorigenesis (Revised Objective #2). Preliminary results from this work indicate that VHL is also an important mediator of normal mammary gland differentiation, confirming that regulation of the hypoxic response is critical for normal mammary gland development. The ultimate goal is of these experiments is to determine the role of HIF-1α over-expression during mammary gland tumorigenesis.

14. SUBJECT TERMS
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Table of Contents

Introduction to the Hypoxic Response and the role of HIF-1α

In response to hypoxia, tissues attempt to restore homeostasis by regulating cellular metabolism and by inducing angiogenesis (reviewed in (Semenza, 2000)]). Both of these processes are primarily regulated by a heterodimeric transcription factor complex known as the Hypoxia Inducible Factor-1, or HIF-1 (Semenza, 2000). The HIF-1 heterodimer includes HIF-1α, a basic helix-loop-helix (bHLH) protein induced and stabilized by hypoxia, and the aryl hydrocarbon receptor nuclear translocator (ARNT) protein (also termed HIF-1β), which is expressed constitutively and heterodimerizes with multiple bHLH partners.

Under normoxic conditions, HIF-1α protein is rapidly degraded through targeted ubiquitination mediated by direct binding of its oxygen dependent domain (ODD) to the β subunit of von Hippel Lindau (VHL) tumor suppressor protein (reviewed in (Kondo and Kaelin, 2001)). In response to hypoxia, HIF-1α protein accumulates, due to decreased interaction with VHL (Krek, 2000). An increase in HIF-1α protein is first detectable at partial pressures of oxygen equivalent to 6% O_2, and becomes maximal between 0.5-1.0% O_2 (Stroka et al., 2001).

In a hypoxic environment, HIF-1 activates the hypoxic response elements (HREs) of target gene regulatory sequences (Huang et al., 1998; Salceda and Caro, 1997), resulting in the transcription of genes implicated in the control of metabolism and angiogenesis, as well as apoptosis and cellular stress (reviewed in (Giordano and Johnson, 2001)). Some of the direct targets include erythropoietin, the angiogenic factor vascular endothelial growth factor (VEGF), glucose transporters and multiple glycolytic enzymes. The connection between the hypoxic response and angiogenesis is also clear from the study of patients with VHL disease, an autosomal, dominantly inherited cancer syndrome. These patients often develop renal clear cell carcinomas

that are massively hypervascular, with highly elevated levels of VEGF expression due to constitutive HIF-1 activity (Kondo and Kaelin, 2001).

REPORT BODY-OBJECTIVE 1

Objective 1: To determine the function of HIF-1α during normal mammary development

Background relevant to Objective #1:

Several laboratories, including our own, have demonstrated that HIF-1α is required to regulate the response to lowered oxygen levels in developing murine tissues (Iyer et al., 1998; Ryan et al., 1998; Schipani et al., 2001; Yun et al., 2002). With respect to known functions of HIF-1, there were several compelling reasons to study HIF-1α function in the context of normal mammary gland development. First, the normal mammary parenchyma undergoes tremendous expansion as it prepares for lactation during the course of pregnancy (Matsumoto et al., 1992), including formation of new blood vessel networks to provide oxygen and nutrients to the lactating mammary gland. For example, in the rat, the vasculature doubles by mid-pregnancy through angiogenesis via sprouting and intussusception (Djonov et al., 2001). Because the developing mammary gland is both highly vascularized and metabolically active with a requirement for glucose to produce milk, it serves as an ideal tissue to determine the *in vivo* role of HIF-1 and its subunit HIF-1α in a developmentally-regulated metabolic switch.

Original Statement of Work Months 4-8 (January 2002-May 2002)

Task 5-Create an established HIF-1α DF mammary epithelial cell line in order to create an epithelial cell line in which HIF-1α can be deleted by treatment with adenovirus expressing Cre recombinase

In order to complete this task, I have purified primary epithelial cells from mid-pregnant HIF *DF* host animals on four independent occasions and have attempted to transfect them with SV40 large T-antigen to create an immortalized cell line. To date, transfection with FuGene, Lipofectamine, or Calcium Phosphate transfection protocols have killed the cells. Because we were able to use primary epithelial cells to observe transcription profiles of various HIF-1α target genes (**Appendix 1-Figure 1**), we do not feel that creation of a stable cell line is necessary to understand the function of HIF-1α in mammary epithelial cells, therefore, we request permission to drop this task from Objective #1.

Original Statement of Work Months 6-18 (March 2002-March 2003)

Task 6-Analyze paraffin-embedded sections prepared from test and control mice over the course of development:

-to confirm the Cre-mediated deletion of HIF-1α

-for histology and H&E-staining

-to determine the percentage of proliferating cells by immunostaining for BrdU

-to determine the percentage of cells undergoing apoptosis by TUNEL staining

-to determine microvessel density by immunostaining with CD31 antibodies

-to determine expression of VEGF, glycolytic enzymes and milk protein gene markers.

At the time of submission of the last annual report, a preprint of the manuscript was enclosed. Additional experiments were required in order for the manuscript to be suitable for publication, including the double staining for Cre and Glut-1 (**Appendix 1-Figure 4C-G**), and Glut-1 immunostaining of the transplanted HIF-1α null cells (**Appendix 1-Figure 7C-D**). As described in this manuscript, all of the experiments proposed under Objective #1, excluding task 5, have been completed. Please refer to the attached manuscript by Seagroves *et al.*, published in April 2003 in *Development* (**Appendix 1**).

Key Research Accomplishments-Objective #1:

- We have confirmed that HIF-1α protein is expressed in purified mammary epithelial cells and is induced by hypoxia
- We have confirmed that the hypoxic response is conserved in mammary epithelial cells
- We have demonstrated a role for HIF-1α in secretory differentiation of alveoli
- We have demonstrated that loss of HIF-1α blocks milk production and impairs milk secretion
- We have demonstrated that loss of HIF-1α impacts milk nutrition
- We have demonstrated that the angiogenesis that occurs during pregnancy during normal mammary gland development is HIF-1α-independent. There were no observed differences in microvessel patterning or density in HIF-1α null glands.
- We have determined that there are no differences in the rates of proliferation or apoptosis in HIF-1α null mammary tissue
- We have discovered the first mouse model to separate mammary epithelial cell proliferation from differentiation

-We have determined that loss of HIF-1α does not impact VEGF transcription *in vivo*.

What does this work mean?

In order to provide a foundation for understanding the role of HIF-1 in mammary tumorigenesis, the function of HIF-1α was investigated during normal mammary gland development under objective #1. Because the majority of human tumors, including breast tumors, contain hypoxic areas, which are more resistant to radiation and chemotherapy (Brown et al. 1998), an enhanced understanding of the molecular mechanisms of HIF-1α function could potentially result in development of new compounds that control mammary epithelial cell fate and/or specifically target hypoxic breast tumor cells.

REPORT BODY-OBJECTIVE 2

Original Objective #2: To determine if deletion of HIF-α results in decreased mammary gland tumor latency, size or burden, or differences in tumor histology/pathology compared to control mice following chemical carcinogen treatment.

PLEASE NOTE THAT WE NOW REQUEST PERMISSION TO CHANGE OBJECTIVE #2 to THE FOLLOWING :

To determine if over-expression of HIF-1α in the mammary gland, achieved through conditional deletion of VHL, results in development of mammary tumors.

Background Relevant to New Objective #2:

Recently, HIF-1α has been demonstrated to be up-regulated in a variety of human solid tumors, in particular breast tumors that exhibit high rates of proliferation (Bos et al., 2001; Zhong et al., 1999). Zhong et al. reported that HIF-1α protein was over-expressed in breast tumors, as well as bordering "normal" areas adjacent to tumors, but not in normal breast tissue (Zhong et al., 1999). These observations in breast tumors are consistent with our previous findings previous that HIF-1α functions as a positive regulator of tumor growth (Ryan et al., 2000; Seagroves and Johnson, 2002). In a subsequent study, the level of HIF-1α expression in breast tumors was correlated with other prognostic factors. Specifically, in ductal carcinoma *in situ* (DCIS) lesions, relatively high levels of HIF-1α expression were associated with increased proliferation as well as increased expression of VEGF and the estrogen receptor (Bos et al., 2001). On the other hand, HIF-1α expression did not correlate with p53 expression, supporting our own laboratory's observations that p53 expression is independent of the effects of loss of HIF-1α on cell growth, metabolism or tumorigenesis (Ryan et al., 2000). Because over-expression of HIF-1α can be achieved by deletion of the VHL protein, conditional deletion of VHL is a powerful method to determine if HIF-1α overexpression contributes to or is a result of tumorigenesis.

Justification for request to change Objective #2:

At the time of submission of the original proposal, we were unsure whether deletion of HIF-1α would accelerate or delay tumorigenesis. We have demonstrated from results obtained through completion of Objective #1 that loss of HIF-1α impairs development of the mammary gland, without impacting proliferation or apoptosis. Therefore, it seems unlikely that loss of HIF-1α will delay tumorigenesis. In addition, given that the expression of Cre driven by either MMTV LTR or

WAP minimal promoters is patchy (Wagner et al. 2001), and mutations induced by DMBA treatment are stochastic, it would be difficult to determine if tumors arose from either HIF-1α wildtype or HIF-1α null cells using a DMBA model as outlined in the original proposal.

More importantly, given the mixed genetic backgrounds upon which these mice are kept (currently a mix of C57BL/6, FVB and 129-SvEv), it will be impossible to perform kidney capsule transplantation of a pituitary isograft into females from male littermates to stimulate constitutive Cre expression without rejection of the isograft. As outlined in the original proposal, for the DMBA treatment paradigm, pituitary isografting is performed on female mice at 5-7 weeks of age. The isografting needs to be scheduled one week prior to the first DMBA treatment in order to stimulate transgene expression prior to carcinogen treatment. Therefore, because any isograft would be rejected by the hosts' immune system, there would be no method available to our laboratory to assure constitutive, expression of Cre in the mammary epithelium of HIF *DF* female mice necessary to delete HIF-1α prior to DMBA treatment.

For these reasons, and given that HIF-1α over-expression has been recently demonstrated in breast tumors, we strongly feel that our new objective #2- to analyze the effects of deletion of the VHL tumor suppressor gene-would be a better model to investigate HIF-1α function during mammary tumorigenesis. In addition, the use of the VHL conditional deletion model will allow our lab to directly test whether or not over-expression of HIF-1α contributes to tumorigenesis, or results from tumor adaptation to hypoxia.

Finally, because a majority of mammary tumor models require multiple pregnancies to stimulate development of mammary tumors, we will also be able to determine if VHL plays an important role in normal mammary gland development by observing the effects of its deletion on the *first round* of pregnancy, lactation and involution.

Revised Statement of Work-New Objective #2

Months 13-31 (October 2002-April 2004)

Task 1-Bred mice to generate test mice (genotype= VHL double-*floxed*, VHL *DF*; Cre-positive) and control females (genotype= VHL *DF*; Cre-negative), using either MMTV-Cre (line D) or WAP-Cre transgenic founders.

Task 2-Perform Western blotting for VHL protein on whole cell extracts prepared from normal mammary gland tissues isolated from wild type mice to determine the expression pattern of VHL.

Task 3-For each Cre transgenic line, biopsy the inguinal pairs of mammary glands from sacrificed test and control mice over the course of mammary development. Two hours prior to biopsy of both inguinal glands, inject all mice with 0.1ml bromodeoxyuridine (BrdU)

-Fix both biopsied glands in neutral buffered formalin for 6h RT

-Utilize 1 gland for whole mount preparation to look for hyperplastic alveolar nodules (HANs)

-Prepare the other gland for paraffin embedding and sectioning to look for epithelial cell disorganization, dysplasia and/or metaplasia

-To analyze nulliparous mice: estimate will require 4 Cre-negative and 4 Cre-positive mice per each Cre transgenic line for a total of 16 mice.

-To analyze pregnant and lactating mice: estimate will require 8 mice per genotype per Cre transgenic line, 4 Cre-negative and 4 Cre-positive, for a total of 16 mice per timepoint to be analyzed, including day 10 of pregnancy, day 15 of pregnancy, day 18 of pregnancy, day 10 of lactation, and day 4 of forced involution, for a total of 80 mice

Task 4-Compare paraffin-embedded sections from test and control mice over the course of development for the first round of pregnancy for the following characteristics:

-proliferation rates by immunostaining for incorporated BrdU

-percentage of cells undergoing apoptosis, by TUNEL technique

-microvessel density by CD31 staining followed by Chalkley counting

-expression of HIF-1α protein, VEGF, PGK-1, Glut-1

Task 5-Begin to constitutively breed another cohort of female mice to attempt to induce mammary tumors by leaving a male with the female and weaning each litter at day 20 of gestation [estimate will require 10-20 mice per genotype per Cre transgenic line for a total of 40-80 mice, depending on frequency of tumor development].

-Biopsy one gland at day 10 of lactation for *each round* of pregnancy

-Prepare glands for whole mount staining or H&E staining to observe development of hyperplasias or tumors

Months 25-36 (October 2003-September 2004):

Task 6-Continue observing the constitutively bred female mice for the appearance of tumors.

Task 7-Record date of onset when tumor first palpable, continue to measure tumor growth by caliper

bi-weekly until tumors reach a pre-determined size, likely to be 1cm x 1cm.

Task 8-Upon tumors growing to the pre-determined size, sacrifice mice, biopsy mammary tumors, fix, prepare for sectioning and archive.

Task 9-Compare time to onset, histology, tumor grade, pathology between test and control mice

Task 10-Determine if areas of pathology correspond to over-expression of HIF-1α protein

Task 11-Determine tumor microvessel density, rates of proliferation and apoptosis

Task 12-Compare expression of HIF-1α target genes in tumors

Task 13-Determine expression levels of HIF-1α and correlate to Cre expression in tumors by double

immunohistochemical staining

Task 14-Determine statistical relevance of results

-tumor-free probability-Kaplan-Meier test, p value <0.05

-time to tumor incidence, chi-square test

-all other tests (for example, percentage of cells proliferating or undergoing apoptosis), student-*t* test, at p <0.05

Task 15-*Alternative approach if no tumors are observed:*

*If no tumors are observed as a result of VHL deletion by month 25, I will begin to cross the VHL *DF*, Cre-positive mice to the MMTV-neu mammary tumor model available from Charles River Laboratories. Approximately 50% of MMTV-neu-positive female mice develop mammary tumors by 6 months of age, approaching 90% penetrance by 1 year of age.

RESULTS

New Objective #2 (Months 13-24):

Since submission of our last annual report, we have obtained the VHL conditional gene deletion mouse model from Jackson Labs, and have bred them to either the MMTV-Cre (line D) or WAP-Cre transgenic mice. We have also completed preliminary, histological analysis of the effects of deletion of VHL upon normal mammary gland development using each Cre transgenic line in the

first round of pregnancy and lactation. Crosses to obtain tissues from multiply bred VHL test and control mice for each Cre transgenic line are also in progress, and up to two rounds of breeding have been completed, however, no tumors have been observed in mice up to 8 months of age.

The results of loss of VHL during the first round of pregnancy are similar between the MMTV-Cre (line D) crosses and the WAP-Cre crosses, therefore, we have focused on the MMTV-Cre crosses to date.

Task 1-Bred mice to generate test mice (genotype= VHL double-*floxed*, VHL *DF*; Cre-positive) and control females (genotype= VHL *DF*; Cre-negative), using either MMTV-Cre (line D) or WAP-Cre transgenic founders.

This task was completed in December 2002. Both VHL *DF* MMTV-Cre (line D)-positive progeny and VHL *DF* WAP-Cre-positive progeny are viable and fertile, as expected.

Task 2-Perform Western blotting for VHL protein on whole cell extracts prepared from normal mammary gland tissues isolated from wild type mice to determine the expression pattern of VHL.

The expression pattern of VHL in the normal mammary gland had not been described at the onset of this project. Therefore, whole cell extracts were prepared from C57BL/6 mice from whole 15-day embryos (15-E, positive control), or from inguinal mammary tissue harvested at the following timepoints: virgin mice, vir; day 6 of pregnancy, 6-P; day 10 of pregnancy, 10-P; day 15 of pregnancy, 15-P; day 10 of lactation, 10-L; and four days following forced involution, 4-Inv (**Appendix 2-Figure 1**). As a control for the increase in epithelial cells that occurs during

mammary gland development, the same blot was stripped and re-probed with anti-cytokertain 8/18 antibodies (CK8/18). VHL protein increased as the number of epithelial cells increased, suggesting that VHL is predominantly expressed in the mammary epithelium. There was a dramatic increase in expression from day 6 to day 10 of pregnancy that was maintained throughout lactation, and decreased during involution. To confirm that VHL was indeed deleted, the bottom panel of Figure 1 shows the expression of VHL in independent wild type (+/+) and two VHL null (-/-) inguinal mammary glands harvested at lactation.

Task 3-For each Cre transgenic line, biopsy the inguinal pairs of mammary glands from sacrificed test and control mice over the course of mammary development. Two hours prior to biopsy of both inguinal glands, inject all mice with 0.1ml bromodeoxyuridine (BrdU)

-Fix both biopsied glands in neutral buffered formalin for 6h RT

-Utilize 1 gland for whole mount preparation to look for hyperplastic alveolar nodules (HANs)

-Prepare the other gland for paraffin embedding and sectioning to look for epithelial cell disorganization, dysplasia and/or metaplasia

I have analyzed mammary gland development at day 15 of pregnancy (15-P), day 18 of pregnancy (18-P) and day 10 lactation (10-L) for MMTV-Cre line D (n=4 Cre-positive; n=4 Cre-positive/timepoint. In VHL null mammary tissue, there is a delay in differentiation at 15-P. All of the alveoli in these glands are collapsed and appear un-differentiated (**Appendix 2-Figure 2**). By day 18 of pregnancy, these alveoli are only partially differentiated (**Appendix 2-Figure 2**). However, compared to the effects observed with deletion of HIF-1α expression as in Objective #1,

even with these subtle changes in alveolar architecture, the VHL null dams are able to nurse their pups and pup growth is normal at first lactation. Some VHL *DF* Cre-positive mice (n=4) have also been observed at the second round of lactation. These glands are less well-differentiated at the second lactation compare to first lactation VHL *DF* Cre-positive dams (**Appendix 2-Figure 3**), but the dams at second lactation are still able to successfully nurse their litters.

Task 4-Compare paraffin-embedded sections from test and control mice over the course of development for the first round of pregnancy for the following characteristics:

-proliferation rates by immunostaining for incorporated BrdU

-percentage of cells undergoing apoptosis, by TUNEL technique

-microvessel density by CD31 staining followed by Chalkley counting

-expression of HIF-1α protein, VEGF, PGK-1, Glut-1

Sections have been archived from pregnant mice injected with BrdU, and BrdU immunostaining has been performed. Cell counting to determine percentage of proliferating cells is in progress. We have also obtained an antibody to HIF-1α from a collaborator at the Burnham Institute that successfully detects murine HIF-1α protein in paraffin-embedded formalin fixed tissues. Using this antibody for immunostaining protocols, we have been able to confirm that, upon deletion of VHL, HIF-1α is indeed over-expressed in mammary epithelial cells, and that the HIF-1 target gene Glut-1 is also over-expressed (**Appendix 2-Figure 4**). In addition, although CD31 immunostaining has yet to be performed, it is evident that loss of VHL results in increased angiogenesis and blood vessel dilation (**Appendix 2-Figure 5**), as expected based on finding in VHL clinical patients.

Task 5-Begin to constitutively breed another cohort of female mice to attempt to induce mammary tumors by leaving a male with the female and weaning each litters at day 20 of gestation [estimate will require 10-20 mice per genotype per Cre transgenic line for a total of 40-80 mice, depending on frequency of tumor development].

-Biopsy one gland at day 10 of lactation for *each round* of pregnancy

-Prepare glands for whole mount staining or H&E staining to observe development of hyperplasias or tumors

Using the WAP-Cre transgenic line to delete VHL, we have constitutively bred mice for up to two rounds of pregnancy. No tumors or HANs have been observed in these mice by either whole mount preparation or H&E staining by the end of second pregnancy, although there are slight changes in epithelial cell morphology and evidence of decreased differentiation similar to that observed in the MMTV-Cre line D line at second lactation (**Appendix 2-Figure 3**).

Task 15-*Alternative approach if no tumors are observed:*

*If no tumors are observed as a result of VHL deletion by month 25, I will begin to cross the VHL *DF*, Cre-positive mice to the MMTV-neu mammary tumor model available from Charles River Laboratories. Approximately 50% of MMTV-neu-positive female mice develop mammary tumors by 6 months of age, approaching 90% penetrance by 1 year of age.

Because no tumors have been observed in the VHL null mammary glands to date, we have recently ordered the MMTV-neu transgenic mammary tumor model from Jackson Labs, and have begun crosses to the VHL *DF*, Cre-positive females in order to generate bi-transgenic females. We will then follow tumor development, as outlined in the revised statement of work. Briefly, tumor latency, burden and pathology will be compared in females that are either VHL *DF*, MMTV-neu-positive and Cre negative to females that are VHL DF, MMTV-neu-positive and MMTV-Cre or WAP-Cre positive. It is anticipated that by the next annual report, the majority of these mice will have developed tumors since latency is approximately six months of age.

Key Research Accomplishments-Objective #2:

- We have confirmed that VHL protein is expressed in the normal murine mammary gland and that expression increases during pregnancy, is maintained at lactation and decreases at involution
- We have demonstrated that VHL regulates differentiation in the normal mouse mammary gland
- We have confirmed that loss of VHL results in over-expression of HIF-1α protein in the mammary epithelium
- We have demonstrated that loss of VHL results in mammary-associated vasculature hypervascularity and dilation

What do these results mean?

Based on these results, we predict that loss of VHL impairs differentiation of the normal murine mammary gland, a novel observation. Therefore, it is clear that both deletion or over-expression of HIF-1α is detrimental to normal mammary gland development, and that the hypoxic response must be tightly regulated in mammary epithelium. But, it is not clear if loss of VHL alone

will result in mammary tumors in aged mice given the relatively few mice observed to date and the young age of the mice. We plan to continue to multiply breed the VHL *DF* Cre-positive mice for each transgenic line, as well as begin breeding the test and control mice into the MMTV-neu mammary tumor model, in order to answer this question.

Summary and Conclusions

We previously demonstrated in our previous annual report a requirement for HIF-1-mediated transcription in the mammary epithelium in order to produce and to secrete milk. These results revealed a novel role for *HIF-1α* in the control of the critical transition from secretory differentiation to secretory activation and of the composition and secretion of milk at lactation.

We now seek to understand how over-expression of HIF-1α, through conditional deletion of VHL, impacts mammary gland development and tumorigenesis. Because VHL is a tumor suppressor gene, we anticipate that loss of VHL will result in mammary tumors. If loss of VHL alone is not sufficient to induce mammary tumors, we anticipate that its loss will accelerate mammary tumorigenesis in the MMTV-neu transgenic mouse mammary tumor model.

Reportable Outcomes since Year 1:

Manuscripts:

Seagroves, T.N., Hadsell, D., McManaman, J., Palmer, C., Liao, D., McNulty, W., Welm, B., Wagner, K.-U., Neville, M. and R.S. Johnson. 2003. HIF-1alpha is a critical regulator of secretory differentiation and activation, but not vascular expansion, in the mouse mammary gland. *Development* **130**: 1713-1724. [please find reprint attached-**Appendix 1**].

Abstracts:

* Oxygen and the Cell, Max Planck Institute, Berlin, Germany, September 2003.

* Keystone Conference, Angiogenesis, Banff, Canada, February 2002.

* Gordon Conference on Mammary Gland Biology, Barga, Italy, 2002.

Presentations:

* Have been invited to be a plenary speaker at the Gordon Conference on Mammary Gland Biology to be held in Il Cicco, Italy, May 2004.

* Department of Defense, Era of Hope meeting, Orlando, FL, 2002.

Funding received based in part on work supported by this award:

NIH renewal of CA82515, awarded to Randall S. Johnson, to support the research costs associated with this project and other projects ongoing in the laboratory to investigate the hypoxic response in a variety of tissue types.

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FIGURE 1-Western Blot , VHL, Normal Mammary Gland Development

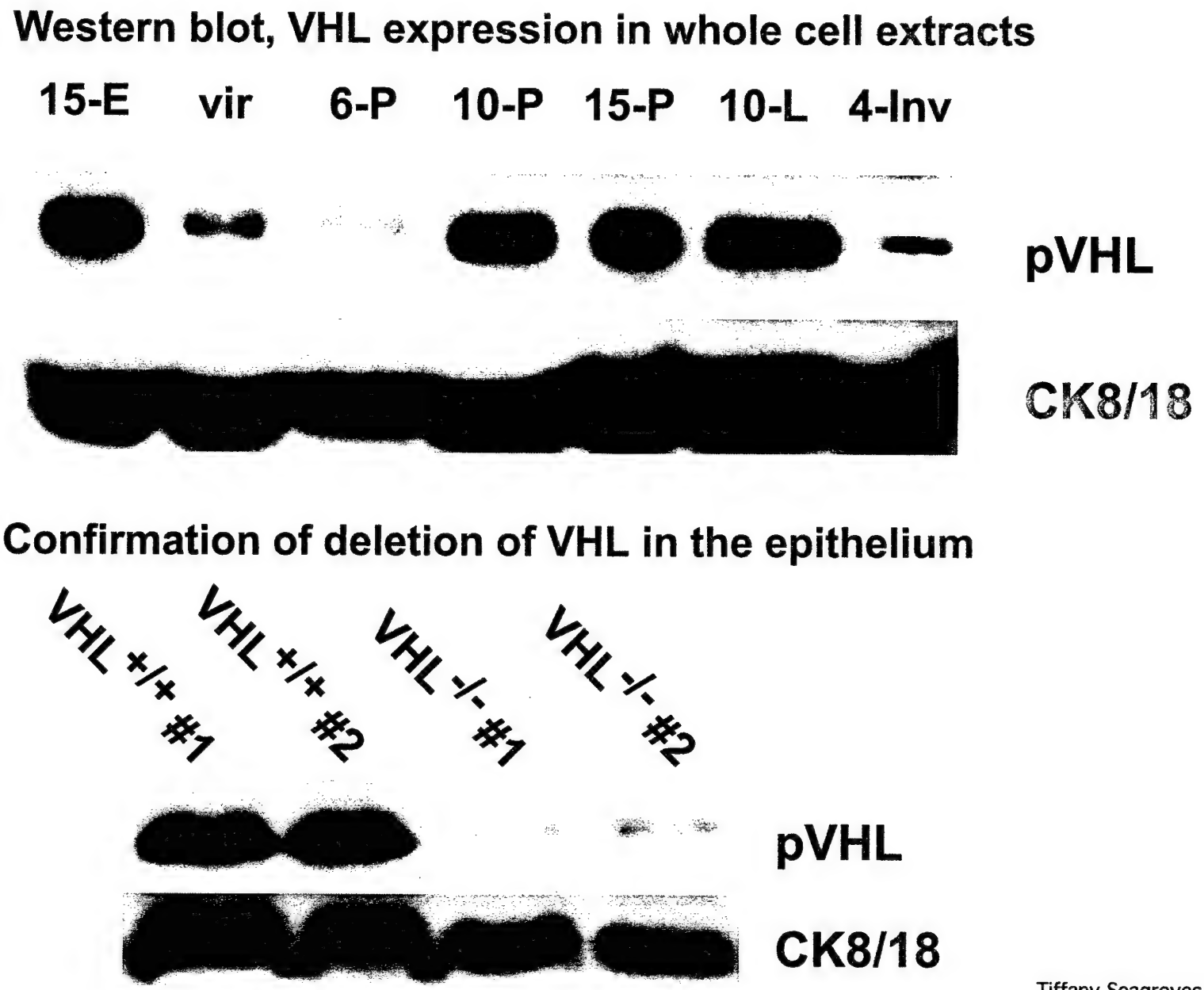

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FIGURE 2-H&E STAINING-MAMMARY GLANDS AT PREGNANCY #1

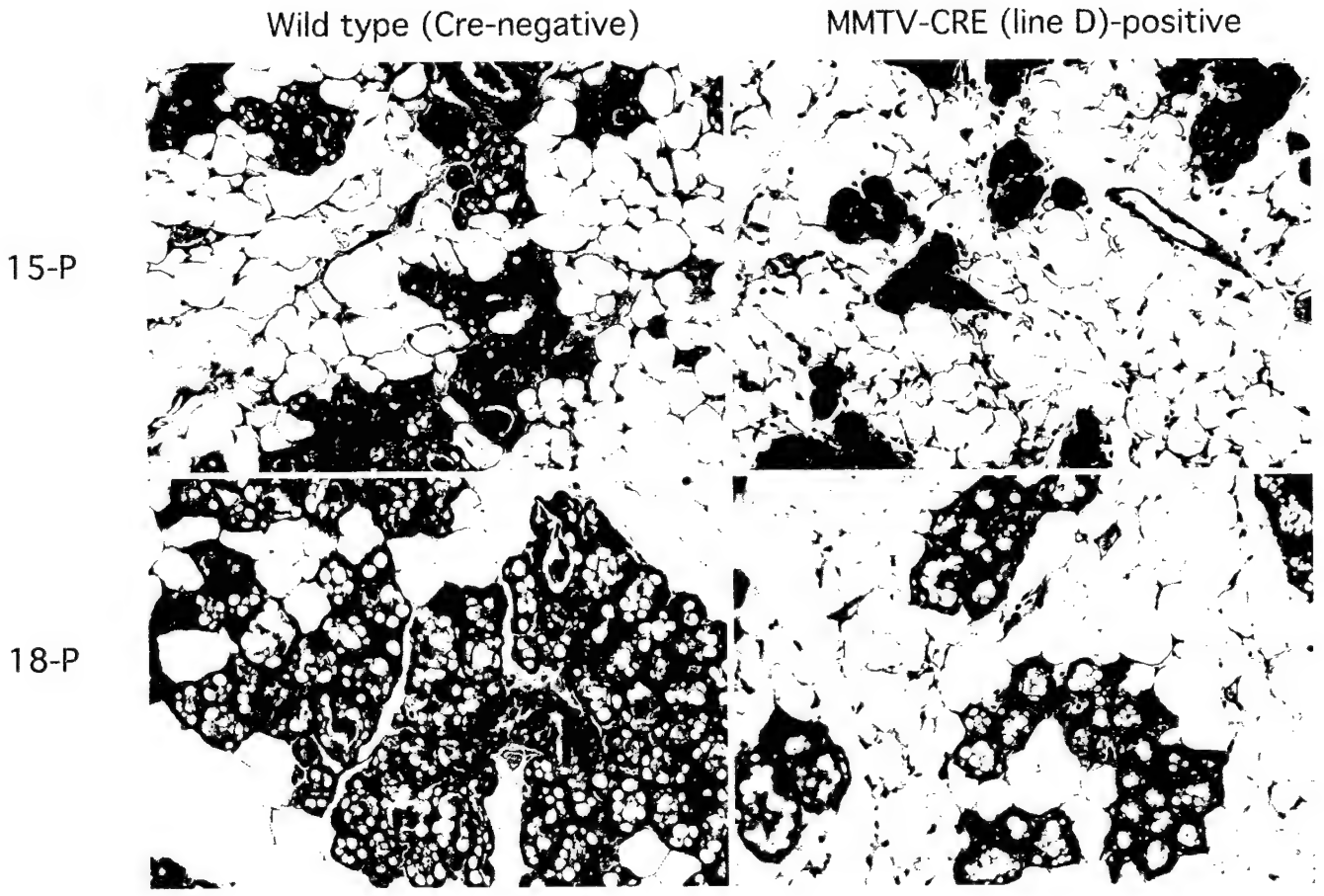

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FIGURE 3-H&E STAINING-MAMMARY GLANDS AT LACTATION #2

Wild type (Cre-negative)

MMTV-CRE (line D)-positive

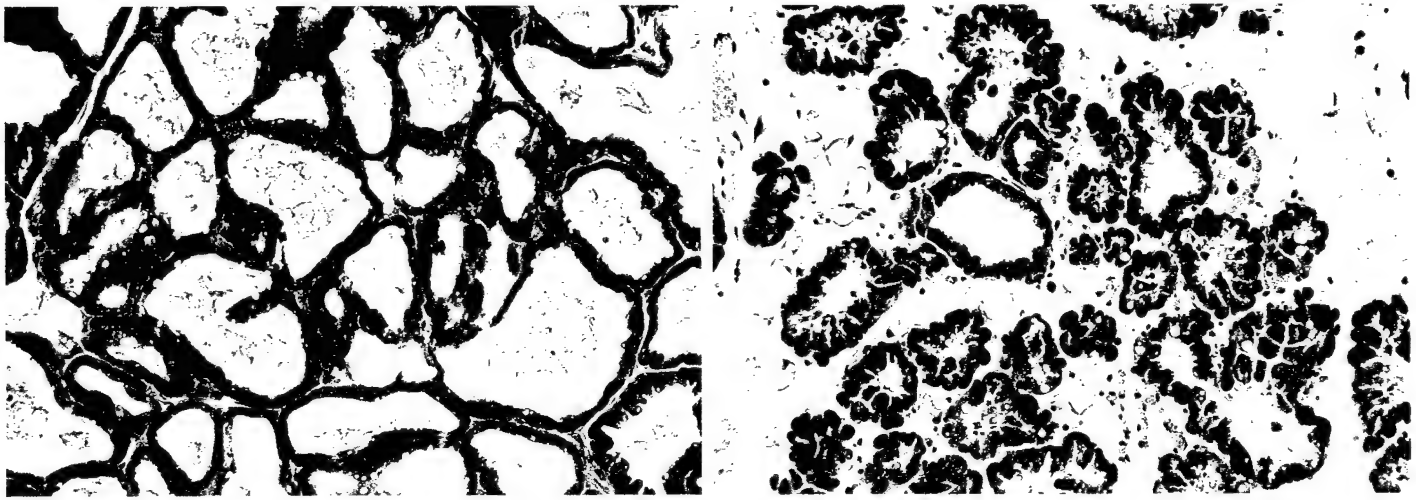

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FIGURE 4- HIF-1alpha and Glut-1 IMMUNOSTAINING AT LACATION #2

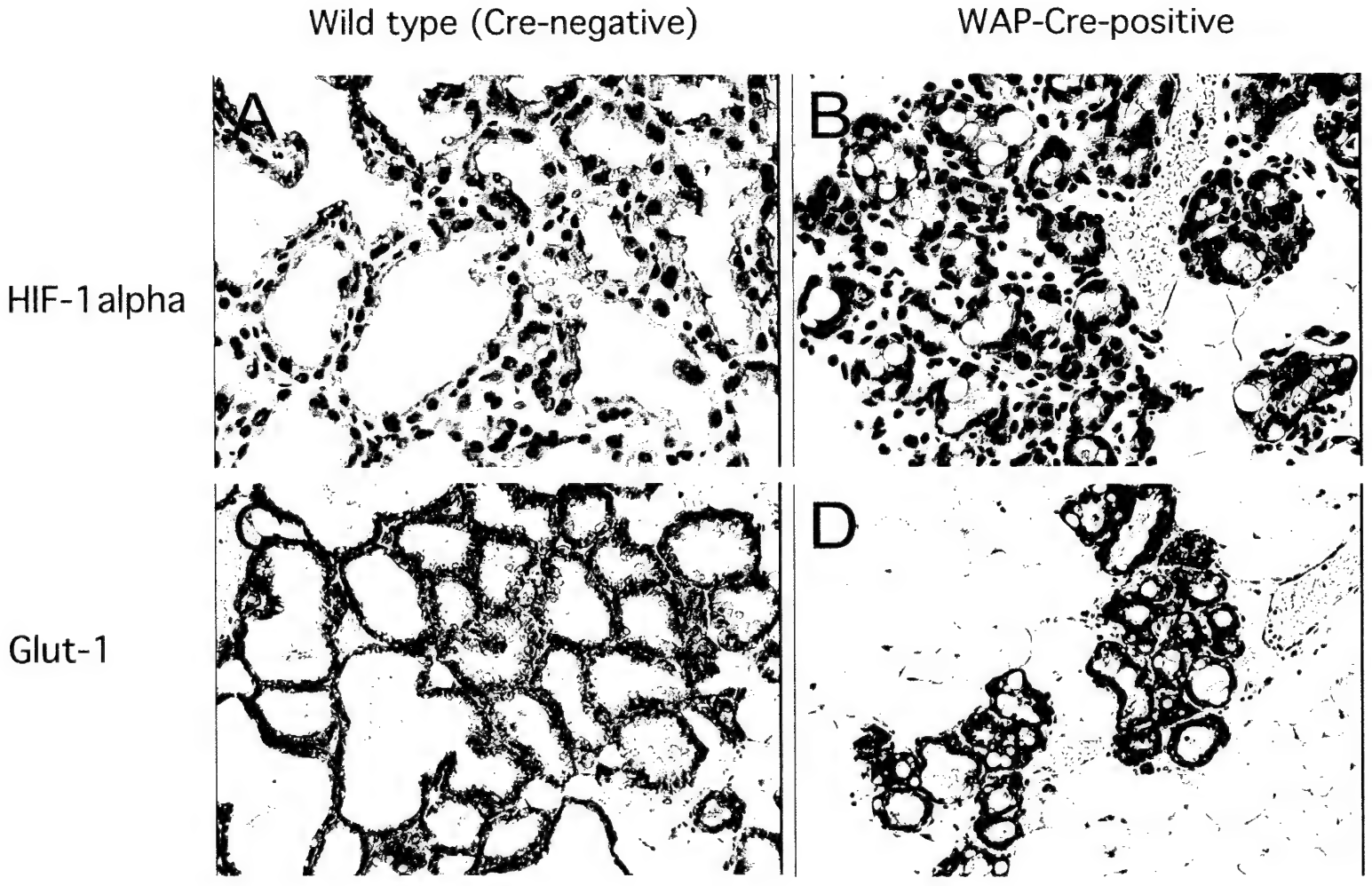

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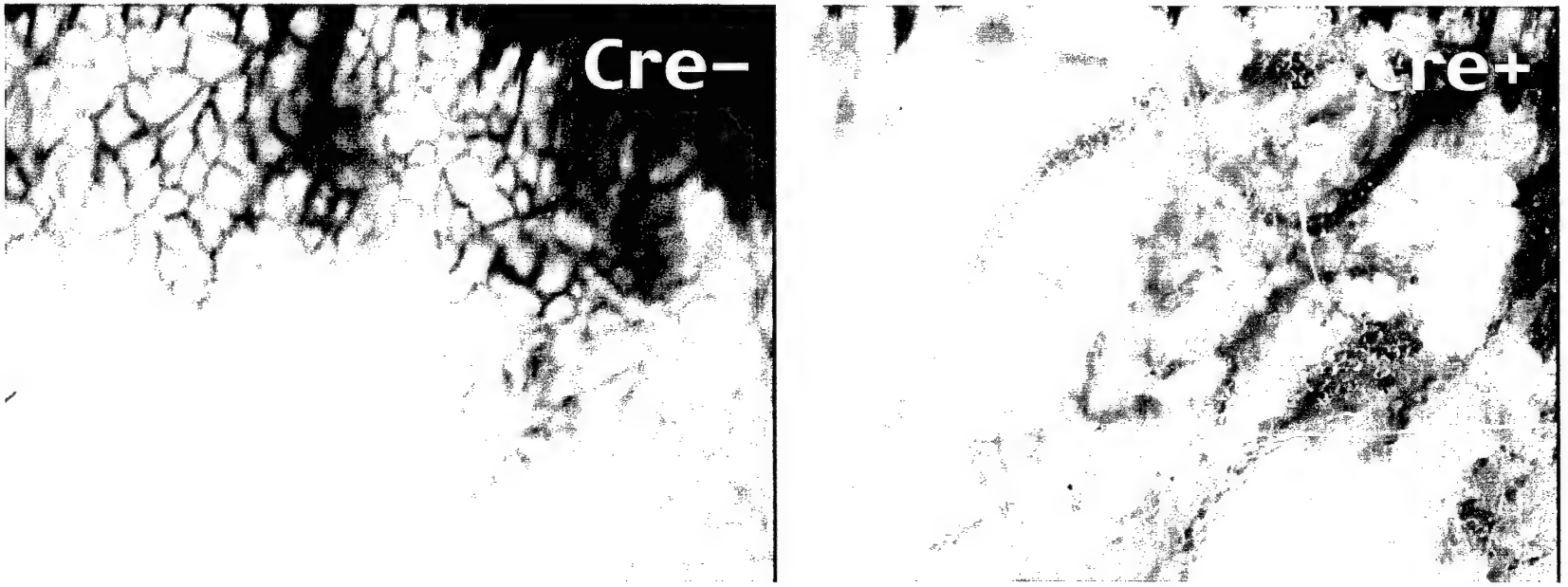
FIGURE 5- HYPERVASCULARITY in VHL NULL GLANDS



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DEVELOPMENT AND DISEASE

HIF1α is a critical regulator of secretory differentiation and activation, but not vascular expansion, in the mouse mammary gland

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SUMMARY

During pregnancy the mammary epithelium and its supporting vasculature rapidly expand to prepare for lactation, resulting in dramatic changes in the microenvironment. In order to investigate the role of oxygenation and metabolism in these processes, the oxygen-responsive component of the hypoxia-inducible factor (HIF) 1 complex, HIF1α, was deleted in the murine mammary gland. Although vascular density was unchanged in the HIF1α null mammary gland, loss of HIF1α impaired mammary differentiation and lipid secretion, culminating in lactation failure and striking changes in milk composition. Transplantation experiments confirmed that these developmental defects were mammary epithelial cell autonomous. These data make clear that HIF1α plays a critical role in the differentiation and function of the mammary epithelium.

Key words: Hypoxia, HIF1, Mammary gland, Lactation, Differentiation, Metabolism, Mouse

INTRODUCTION

In response to hypoxia, tissues attempt to restore homeostasis by regulating cellular metabolism and by inducing angiogenesis (reviewed by Semenza, 2000). Both of these processes are primarily regulated by a heterodimeric transcription factor complex known as the hypoxia inducible factor 1 or HIF1 (Semenza, 2000). The HIF1 heterodimer includes HIF1α, a basic helix-loop-helix (bHLH) protein induced and stabilized by hypoxia, and the aryl hydrocarbon receptor nuclear translocator (ARNT) protein (also termed HIF1β), which is expressed constitutively and heterodimerizes with multiple bHLH partners.

Under normoxic conditions, HIF1α protein is rapidly degraded through targeted ubiquitination mediated by direct binding of its oxygen dependent domain to the β subunit of the von Hippel Lindau (VHL) tumor suppressor protein (reviewed by Kondo and Kaelin, 2001). In response to hypoxia, HIF1α protein accumulates, owing to decreased interaction with VHL (Krek, 2000). An increase in HIF1α protein is first detectable at partial pressures of oxygen equivalent to 6% O_2, and is maximal between 0.5-1.0% O_2 (Stroka et al., 2001). In a hypoxic environment, HIF1 activates the hypoxic response elements (HREs) of target gene regulatory sequences (Huang et al., 1998; Salceda and Caro, 1997), resulting in the transcription of genes implicated in the control of metabolism and angiogenesis, as well as apoptosis and cellular stress (reviewed by Giordano and Johnson, 2001). Some of the direct targets include erythropoietin, the angiogenic factor vascular endothelial growth factor (VEGF), glucose transporters and multiple glycolytic enzymes. The connection between the hypoxic response and angiogenesis is also clear from the study of individuals with VHL disease, an autosomal, dominantly inherited cancer syndrome. Individuals heterozygous for one inactivating mutation in *VHL* are predisposed to developing a variety of tumor types, including renal clear cell carcinomas that are massively hypervascular with highly elevated levels of VEGF expression caused by constitutive HIF1 activity in response to inactivation of the second *VHL* allele (Kondo and Kaelin, 2001).

Recently, HIF1α has been demonstrated to be upregulated in a variety of human solid tumors, in particular breast tumors that exhibit high rates of proliferation (Bos et al., 2001; Zhong et al., 1999). Zhong et al. reported that HIF1α protein was overexpressed in breast tumors, as well as bordering 'normal' areas adjacent to tumors, but not in normal breast tissue (Zhong et al., 1999). These observations in breast tumors are consistent with our previous findings that HIF1α functions as a positive

regulator of tumor growth (Ryan et al., 2000; Seagroves and Johnson, 2002). In a subsequent study, the level of HIF1α expression in breast tumors was correlated with other prognostic factors. Specifically, in ductal carcinoma in situ (DCIS) lesions, relatively high levels of HIF1α expression were associated with increased proliferation, as well as increased expression of VEGF and the estrogen receptor (Bos et al., 2001). However, HIF1α expression did not correlate with p53, supporting the observations of our own laboratory that the effects of loss of *Hif1a* on cell growth, metabolism or tumorigenesis are independent of p53 expression (Ryan et al., 2000). In order to provide a foundation for understanding the role of HIF1 in mammary tumorigenesis, the function of HIF1α was investigated during normal mammary gland development.

Several laboratories have demonstrated that HIF1α is required to regulate the response to lowered oxygen levels in developing murine tissues (Iyer et al., 1998; Ryan et al., 1998; Schipani et al., 2001; Yun et al., 2002). With respect to known functions of HIF1, there were several compelling reasons to study HIF1α function in the context of normal mammary gland development. First, the normal mammary parenchyma undergoes tremendous expansion as it prepares for lactation during the course of pregnancy (Matsumoto et al., 1992), including formation of new blood vessel networks to provide oxygen and nutrients to the lactating mammary gland. For example, in the rat, the vasculature doubles by mid-pregnancy through angiogenesis via sprouting and intersucception (Djonov et al., 2001).

In addition, in preparation for lactation, there is a requirement for glucose to provide energy as well as to synthesize lactose, the primary carbohydrate in milk. Notably, the increased activity of several glycolytic enzymes involved in glucose metabolism has been reported at the transition from pregnancy to lactation (Mazurek et al., 1999). The transition from differentiation during pregnancy to successful milk secretion at lactation is complex, and has been divided into two stages, recently termed secretory differentiation and secretory activation (McManaman and Neville, 2003). Secretory differentiation begins at mid-gestation with the production of significant quantities of milk protein and lipid. Secretory activation is coordinated with the birth of pups, and depends on the completion of secretory differentiation. The increased demands for energy for synthesis of milk components that begin during pregnancy persist during lactation, as the gland is actively making and secreting milk. Because the developing mammary gland is both highly vascularized, and metabolically active, with a requirement for glucose to produce milk, it serves as an ideal tissue to determine the in vivo role of HIF1 and its subunit HIF1α in a developmentally regulated metabolic switch.

The clear increase in demands for energy during lactation, as well as the striking and extensive angiogenesis that occurs during pregnancy, led us to hypothesize that during both secretory differentiation and activation, HIF1α may be required to alleviate transient hypoxia through angiogenesis, increased dependence on glycolysis and regulation of substrates for the production of milk. In order to test this hypothesis, we have specifically removed *Hif1a* from the mammary epithelium using previously characterized HIF 'floxed' mice (Ryan et al., 2000) that express MMTV-Cre (Wagner et al., 2001; Wagner et al., 1997). In these mice, multiple facets of the differentiation process were impaired, culminating in a functional failure of the mammary gland.

MATERIALS AND METHODS

Animals and tissue collection

Animals were housed in an AAALAC-approved facility in filter-topped cages and provided with food and water ad libitum. All animal experiments were conducted using the highest standards for humane care in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Mice harboring two alleles of exon 2 of *Hif1a* flanked by loxP sites (*HIF1a*$^{f+/f+}$ or 'floxed') (Ryan et al., 2000) were bred to MMTV-Cre (line A) transgenic mice (Wagner et al., 2001). Wild type (*HIF1a*$^{f+/f+}$, MMTV-Cre-negative) and HIF1α 'null' (*HIF1a*$^{f+/f+}$, MMTV-Cre-positive) littermate females were bred with CD-1 males (day of plug=0). Mammary glands were harvested from mice at day 10 (n=3/genotype), day 15 (n=6/genotype) and day 18 of pregnancy (n=5/genotype), day 1 of lactation (date of birth, n=4/genotype), or day 10 of lactation (n=>25/genotype). At sacrifice, one inguinal gland was fixed for 6 hours at room temperature with 10% neutral buffered formalin (NBF) prior to paraffin wax embedding, sectioning and staining with Hematoxylin and Eosin.

Quantitation of DNA, RNA and protein

A piece of inguinal mammary gland harvested at day 18 of gestation was finely ground to a powder under liquid nitrogen and homogenized in a modified RIPA buffer (50 mM Tris, pH 7.4, 1% NP-40, 0.25% sodium desoxycholate, 400 mM NaCl, 1 mM EDTA in RNase-free water). DNA was quantitated after Hoechst 33258 staining using a Horiba Micromax fluorometer (excitation, 350 nm; emission 473 nm). To measure RNA, homogenates were first treated for 90 minutes with DNase I, and the fluorescence intensity quantitated following incubation with Ribogreen Dye (Molecular Probes). Protein was measured using a Bradford assay (BioRad).

Milk collection and analysis

At birth pups were removed from their natural mothers, randomized and 10-12 pups placed with each dam. The average pup weight per litter per day was determined until mid-lactation (day 9-11 lactation), when milk and mammary tissues were collected. Milk was collected under gentle vacuum into tared tubes on ice from weaned dams injected with oxytocin (1.5 U per leg, i.m.). For each sample, the water, fat, nitrogen, lactose, sodium and chloride contents were measured according to standard protocols (Jensen, 1995). Briefly, the percentage of water (% w/w) was measured as weight loss after drying, and sodium and chloride were measured by inductively coupled plasma spectrometry using a Spectro-CIROS^{CCD} (Spectro Analytical Instruments). To compare wet weight of the lactating glands, both inguinal glands were dissected following milking, flash frozen and weighed immediately.

Primary culture, adenoviral infection and transplantation

Primary mammary epithelial cells (MEC) were isolated from *Hif1a*$^{f+/f+}$ pregnant mice according to Pullan and Streuli (Pullan and Streuli, 1997). Equal volumes of cells were allowed to spread onto plastic dishes in plating medium (Ham's F12 containing 10% FBS, 5 μg/ml insulin, 1 μg/ml hydrocortisone, 20 ng/ml murine epidermal growth factor, 5 ng/ml cholera toxin and 50 μg/ml gentamicin, 100 U Penincillin/10U Streptomycin) for 48 hours before replacing this medium with growth medium (same as plating, but no cholera toxin and 5% FBS). The next day, the cells were infected overnight with either Adenovirus-β-galactosidase (Adeno-βgal) or Adenovirus-Cre (Adeno-Cre, generously provided by Dr Frank Giordiano) at a multiplicity of infection of 60-65 particles per cell (Rijnkels and Rosen, 2001). The next day, the cells washed several times with PBS and fresh growth medium was added. Cells were allowed to recover from infection for 24-48 hours. To compare mRNA expression of target genes of cultured MEC or to prepare nuclear extracts for western blotting, the medium was changed to growth medium

containing 25 mM HEPES pH 7.4 at 0 hours. Cells were then left at normoxia or transferred to a hypoxic incubator (0.5% O_2 balanced with N_2) for 24 hours. For transplantation into host mice, cells were trypsinized, washed and resuspended to 50,000-100,000 cells/μl in HBSS. Approximately 10-15 μl of cells were injected into the cleared inguinal fat pads of 3-week old immunocompromised *Rag1*$^{-/-}$ females (Jackson Labs). After a period of MEC outgrowth of at least 10 weeks, the hosts were then mated and the outgrowths (4R, Adeno-β-gal-infected, wild type; 4L, Adeno-Cre infected, *Hif1a*$^{-/-}$) harvested and fixed in 10% NBF.

Nuclear extract preparation and western blotting

Nuclear extracts (NE) were prepared as described previously (Ryan et al., 1998). HIF1α protein was detected by western blotting using 60 μg input of NE resolved by a 6% SDS-PAGE gel and transferred to PVDF membrane. The membrane was blocked overnight at 4°C in 10% nonfat dry milk, followed by a 3 hour incubation in a 1:1000 dilution of anti-mouse HIF1α antibody (Novus, NB100-123). The blot was then incubated in a 1:10,000 dilution of anti-mouse whole IgG-HRP for 30 minutes followed by incubation in ECLPlus substrate (Amersham) prior to exposure to Kodak MR film.

Preparation of RNA and DNA

Cells were washed twice with cold PBS before being directly extracted with RNAzol B for total RNA preparation (Tel-Test) or scraped into buffer containing proteinase K for preparation of genomic DNA. To prepare total RNA from tissues, snap-frozen tissue was pulverized with a mortar and pestle directly in liquid nitrogen and homogenized in chilled RNAzol B and RNA prepared according to manufacturer's instructions.

Immunostaining

For CD31 staining and Chalkley analysis slides were processed as previously described (Ryan et al., 2000). Slides were blinded and ten 10× power fields counted twice independently (n=5 per genotype). Anti-Cre immunostaining was performed as previously described (Seagroves and Li, 2002). For Glut1, antigen retrieval was performed on paraffin wax sections using 1× citrate buffer (DAKO) followed by overnight incubation at room temperature of a 1:200 dilution of anti-Glut1 antibody (Alpha Diagnostics). Staining was visualized via the ABC Elite staining kit (Vector Laboratories) using DAB as a substrate followed by counterstaining with Hematoxylin. For double labeling to detect both Glut1 and Cre on the same paraffin wax sections, Glut1 was first detected as described using DAB substrate. The sections were then re-blocked with 10% goat serum and Cre detected using Vector VIP (purple color) substrate followed by counterstaining with Methyl Green.

Semi quantitative reverse transcription PCR assays

Random-primed reverse transcription was carried out on 30 ng of total RNA. The cDNA was amplified using primers to mouse *Xor*, α-lactalbumin, β-casein, adipophilin (*Adfp*), butyrophilin and β-actin genes. Samples were prepared for loading onto the Applied Biosystems 310 Genetic Analyzer by mixing 12 μl of formamide, 1 μl of TAMARA size standard (Perkin Elmer Applied Biosystems) and 2 μl of PCR product. The size and amount of PCR product was calculated using GeneScan software (Perkin Elmer Applied Biosystems). Control experiments were performed to define signal linearity for each probe pair.

Real-time PCR assays

Two micrograms of total RNA was DNase I treated and directly used to prepare first-strand cDNA from random hexamer primers using the Superscript II Reverse Transcription Kit (Invitrogen). For real-time detection PCR (RTD-PCR), 5 ng of input cDNA was analyzed in triplicate per primer pair per sample and the corresponding threshold cycle (Ct) values expressed as the mean±s.e.m. All reactions were performed using 2× Taq Master Mix (Perkin Elmer Applied Biosystems), 900 nM each of the forward and reverse PCR primers and 250 nM of a fluorescently tagged primer pair-specific probe in a total volume of 25 μl using default cycling parameters on an ABI Prism 7200 Sequence Detector. The following primer and probe sequences were used.

PGK1: (F) 5′-CAGGACCATTCCAAACAATCTG-3′; (R) 5′CTGT-GGTACTGAGAGCAGCAAGA-3′; (probe) 5′-(6~FAM)TAGCTCG-ACCCACAGCCTCGGCATAT-(TAMRA)-3′.

Glut1: (F) 5′-ACGAGGAGCACCGTGAAGAT-3′; (R) 5′-GGGCAT-GTGCTTCCAGTATGT-3′; (probe) 5′-(6~FAM)CAACTGTGCGGC-CCCTACGTCTTC-(BHQ)-3′.

VEGF total: (F) 5′-ATCCGCATGATCTGCATGG-3′; (R) 5′-AGT-CCCATGAAGTGATCAAGTTCA-3′; (probe) 5′-(6~FAM)TGCCC-ACGTCAGAGAGCAACATCAC-(BHQ)-3′

Claudin 7: (F) 5′-CGAAGAAGGCCCGAATAGCT-3′; (R) 5′-GCT-ACCAAGGCAGCAAGACC-3′; probe 5′-(6-FAM)-GCCACAATG-AAAACAATGCCTCCAGTCA-(BHQ)-3′

Claudin 8: (F) 5′-TGGTGGATGTGGCCCTAAA-3′; (R) 5′-CGC-TGTGGTCCAGCCTATGT-3′; probe: 5′-(6-FAM)-GAGGGCTTC-TCCCAGCTCGCG-(BHQ)-3′

CK19: (F) 5′-CCCTCCCGAGATTACAACCA-3′; (R) 5′-TGGTG-GCACCAAGAATCTTG-3′; (probe) 5′-(6-FAM)-CTTTAAGACCA-TCGAGGACTTGCGCG-(BHQ)-3′

Normalization of real-time PCR assays

In cultured cells, target gene mRNA expression was first normalized to 18S rRNA and then expressed as a percentage of signal observed in wild-type cells (*Hif1a*$^{f+/f+}$, Adeno-β-gal-infected) cultured at normoxia (onefold). For mammary gland tissue samples, expression of each sample was first normalized to cytokeratin 19 (*Ck19*), a gene exclusively expressed in epithelial cells (Nagle et al., 1986), to correct for any differences in epithelial cell content between genotypes prior to comparison of gene induction between wild-type (*Hif1a*$^{f+/f+}$ only; MMTV-Cre negative) and null (*Hif1a*$^{f+/f+}$, MMTV-Cre-positive) glands. Expression of each target in null glands was determined relative to the signal observed in wild type samples (onefold) according to standard procedures [ABI Prism 7200 manual and Muller et al. (Muller et al., 2002)]. Each primer set approached 100% amplification efficiency, allowing direct comparison of Ct values to determine relative gene expression (Muller et al., 2002). To determine the efficiency of deletion of *Hif1a*, genomic DNA was prepared and the expression level of *Hif1a* was compared with that of a control primer set, *Jun*.

Vasculature labeling

Fluorescein-conjugated lectin from tomato (Vector Laboratories) diluted to 1mg/ml in PBS was injected into the tail vein of live mice 5 minutes prior to perfusion. One minute prior to perfusion, mice were anesthetized and 1% paraformaldehyde (PFA)/0.5% glutaraldehyde was perfused directly in the heart at a rate of 1 ml per minute followed by clearing with PBS. Mammary tissues were equilibrated in cold 30% sucrose/PBS for 2 hours before embedding in OCT compound on dry ice. Thick frozen sections (40-50 μm) were post-fixed in 4% PFA for 5 minutes, treated with 0.5% Triton-X for 10 minutes, and incubated with AlexaFluor 595-conjuated phalloidin (Molecular Probes) at a 1:250 dilution in 1% BSA/PBS for several hours prior to mounting. A Zeiss confocal microscope was used to capture 0.5 μm serial slices at low power, which were then merged into one plane to visualize the vasculature (green) and actin filaments of the epithelial network (red).

Statistical analysis

Statistical significance was determined by an unpaired t-test (P set to <0.05), using StatView 5.0 (SAS). Those samples that achieved statistical significance, comparing wild-type with *Hif1a*$^{-/-}$ samples, are indicated with an asterisk.

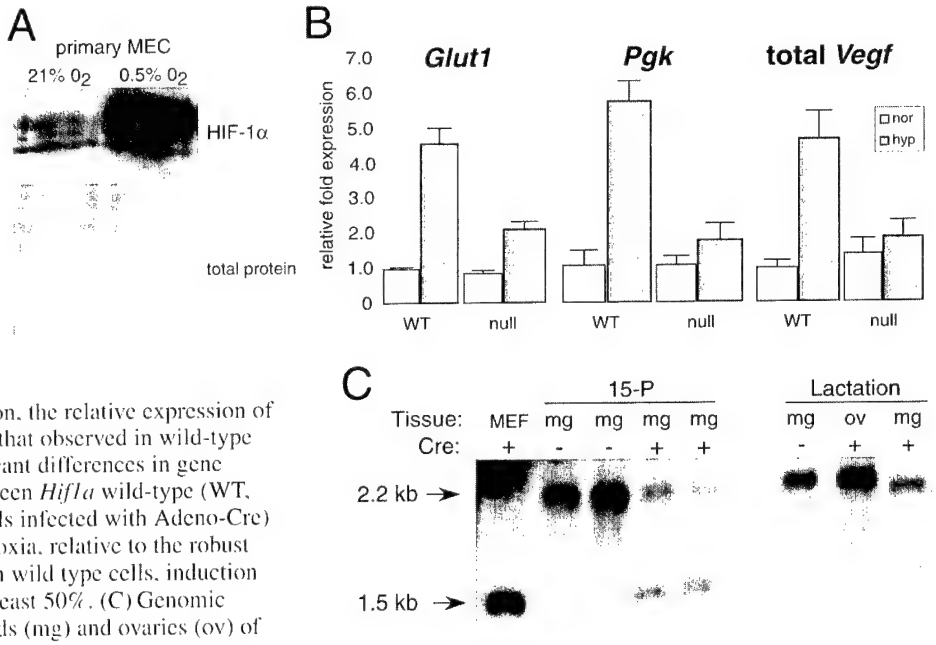

Fig. 1. Mammary epithelial cells contain functional HIF1. (A) Upper panel: HIF1α, a triplet present at ~120 kDa, was detected in purified mammary epithelial cells (MEC) at normoxia (21% O_2) and was induced dramatically upon exposure to 0.5% oxygen. Lower panel: to demonstrate equivalent loading, total protein was stained with a reversible protein detection dye prior to blotting. (B) Purified MEC were cultured at either normoxia (white bars) or hypoxia (gray bars), harvested, and expression of target genes was normalized to 18S rRNA. After normalization, the relative expression of each gene was expressed as a percentage of that observed in wild-type cells at normoxia (mean±s.e.m.). No significant differences in gene expression were observed for any gene between *Hif1a* wild-type (WT, $Hif1a^{f+/f+}$ only) and null cells ($Hif1a^{f+/f+}$ cells infected with Adeno-Cre) cultured under normoxic conditions. At hypoxia, relative to the robust induction of *Pgk*, *Glut1* and *Vegf* observed in wild type cells, induction of all of these mRNAs was decreased by at least 50%. (C) Genomic DNA was prepared from the mammary glands (mg) and ovaries (ov) of $Hif1a^{f+/f+}$ (Cre-negative) or $Hif1a^{f+/f+}$, MMTV-Cre-positive mice at either day 15 of gestation (15-P) or at mid-lactation and used for Southern blotting as described previously (Ryan et al., 1998). As a control for complete excision, DNA was prepared from primary mouse embryonic fibroblasts (MEF) previously infected with Adeno-Cre.

RESULTS

HIF1α is expressed in primary mammary epithelial cells and is required for hypoxia-inducible gene transcription

To determine if HIF1α is expressed in normal murine mammary epithelium, mammary epithelial cells were purified from wild-type mid-pregnant C57BL/6:129-Sv mice, cultured under hypoxic conditions and the extracts probed for HIF1α protein. In contrast to previous reports that HIF1α is undetectable in normal human breast tissue (Zhong et al., 1999), low levels of HIF1α protein were detectable in nuclear extracts of purified murine MEC cultured at normoxia (21% O_2). Robust induction of HIF1α was observed in response to culture under hypoxic conditions (Fig. 1A).

To determine if HIF1 is essential for hypoxia-induced transcription in mammary epithelial cells, the expression levels of *Pgk* (*Pgk1* – Mouse Genome Informatics), *Glut1* (*Slc2a1* – Mouse Genome Informatics) and *Vegf* (*Vegfa* – Mouse Genome Informatics) were compared in primary cultures of wild-type and $Hif1a^{-/-}$ cells cultured at normoxia or hypoxia by real-time detection PCR (RTD-PCR, Fig. 1B). Under hypoxia loss of *Hif1a* resulted in minimal induction of *Pgk* mRNA and the hypoxia-inducible expression of both *Glut1* and total *Vegf* was reduced by at least 50%. These results demonstrated that regulation of hypoxic response acts through HIF1 in mammary epithelium.

Conditional deletion of HIF1α in the mouse mammary gland

To determine whether HIF1 function is required in vivo for normal mammary gland development, a conditional gene deletion strategy was employed to delete *Hif1a* in the mammary epithelium of mice. Mice harboring two 'floxed' alleles of exon 2 of the *Hif1a* locus ($Hif1a^{f+/f+}$) (Ryan et al., 2000) were bred with $Hif1a^{f+/f+}$ mice that expressed Cre under control of the mouse mammary tumor virus (MMTV)-LTR, which targets deletion in the mammary epithelium, but not in the stroma. $Hif1a^{f+/f+}$ progeny negative for MMTV-Cre (referred to as wild type) were compared with $Hif1a^{f+/f+}$ littermates that expressed MMTV-Cre. The temporal-spatial pattern of Cre recombinase activity in this line of mice (line A) has been extensively described (Wagner et al., 2001). Southern blot analysis comparing wild-type and $Hif1a^{-/-}$ mammary tissue indicated that MMTV-Cre consistently targeted deletion of *Hif1a* in at least 50% of the epithelial cells (Fig. 1C). However, because the DNA was prepared from whole tissue, the intensity of recombined allele, which is only present in the epithelial cells, is actually underestimated by Southern blotting. As described in detail by Wagner et al. using *ROSA* reporter mice crossed to individual lines of MMTV-Cre transgenic mice, by lactation, the majority of the epithelial cells have been targeted for recombination (Wagner et al., 2001). Therefore, based on these previous observations, as well as the data generated by Southern blotting, the mammary glands isolated from $Hif1a^{f+/f+}$, MMTV-Cre-positive mice will be referred to as *Hif1a* null ($Hif1a^{-/-}$). Furthermore, in contrast to reports that this line of MMTV-Cre transgenic mice induced excision in the ovaries of mature mice (Wagner et al., 2001), no recombination of the *Hif1a* locus could be detected in DNA prepared from whole ovaries of $Hif1a^{f+/f+}$, MMTV-Cre-positive females either by Southern blotting (Fig. 1C) or by RTD-PCR.

During pregnancy deletion of HIF1α impairs secretory differentiation, but not vascular expansion

In order to pinpoint the stage of mammary gland development at which HIF1α function may be required, mammary tissue

was harvested from mice over the course of gestation. No differences in ductal morphogenesis were noted between genotypes in nulliparous mice (data not shown). At day 10 of gestation, a stage of development prior to differentiation, no defects in histology were observed in *Hif1a*$^{-/-}$ glands at either the gross or microscopic level (data not shown), indicating that HIF1α is not crucial for early rounds of alveolar proliferation. Similarly, by day 12 of pregnancy, when secretory differentiation typically begins in most mouse strains, no differences in morphology were noted (data not shown).

However, by day 15 of pregnancy, well into the period of secretory differentiation, although the glands of both genotypes were indistinguishable at the whole-mount level (data not shown), histological examination revealed significant abnormalities in the *Hif1a*$^{-/-}$ glands (Fig. 2A,B). In particular, the protein and lipid droplets that give the wild-type epithelium a 'lacy' appearance were completely absent in the *Hif1a*$^{-/-}$ glands. In addition, null alveoli were smaller, with reduced lumens, and the surrounding connective tissue that normally regresses as the alveoli mature was more prominent than in wild-type glands. These defects resulted from a block in differentiation, rather than proliferation, as the rates of incorporation of bromodeoxyuridine were equivalent at this stage of development (data not shown).

Because vascular density doubles over the course of rodent mammary development, and HIF1 has been implicated in the control of angiogenesis (Forsythe et al., 1996) we next analyzed the effect of deletion of HIF1 on vasculogenesis. Vessels were visualized by injection of fluorescein-conjugated tomato lectin into the tail veins of live mice at day 15-16 of pregnancy (Fig. 2C,D). Surprisingly, no gross differences in microvessel patterning, or vascular density were observed in *Hif1a*$^{-/-}$ mammary glands.

Expression profiles of HIF1 targets and markers of differentiation in pregnant mice

The expression of HIF1 targets, normalized to the epithelial cell marker cytokeratin 19 (*Ck19*; *Krt1-19* – Mouse Genome Informatics), was then analyzed in mammary glands of pregnant mice at day 15 of gestation. In contrast to cultured primary cells exposed to hypoxia, neither *Pgk* nor *Vegf* mRNA expression differed significantly between genotypes (Fig. 3A). However, *Glut1* expression was decreased by 60%. During secretory differentiation, transcription of markers associated with milk production increase sharply; therefore, to further characterize the defects in differentiation in the epithelial cells, a panel of markers associated with production of milk components was compared using semi-quantitative RT-PCR following normalization to β-actin (Fig. 3B). Two of these markers, β-casein and α-lactalbumin (α-lac), are markers of the casein and whey fraction of milk, respectively. In addition, several markers were analyzed that are associated with the milk lipid globule (MLG). These included xanthine oxidoreductase (XOR; XDH – Mouse Genome Informatics), a redox enzyme immunolocalized to the apical plasma membrane of lactating

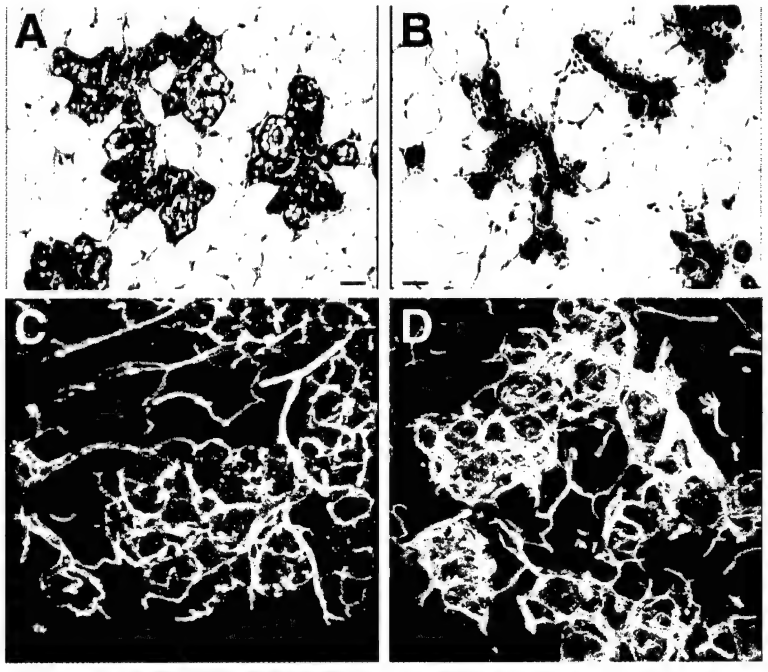

Fig. 2. Defects in secretory differentiation, but not vascular expansion, at day 15 of pregnancy. (A,B) Paraffin wax-embedded sections prepared from wild-type (A) or *Hif1a*$^{-/-}$ (B) glands isolated at day 15 of pregnancy stained with Hematoxylin and Eosin. Scale bar: 50 μm. Note the striking block in differentiation in the *Hif1a*$^{-/-}$ glands (B). (C,D) Patterning of the vasculature in relationship to the mammary epithelium after lectin (green) and phalloidin (red) staining in wild-type (C) and *Hif1a*$^{-/-}$ glands (D).

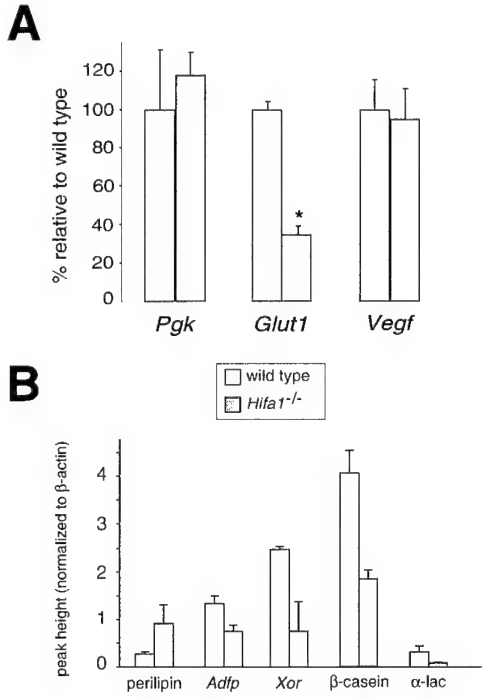

Fig. 3. Analysis of markers at day 15 of pregnancy. (A) RTD-PCR was used to compare expression of HIF1 target genes following normalization to *Ck19* (mean±s.e.m.). (B) The mRNA expression levels of several known markers of mammary epithelial cell differentiation, including β-casein, α-lactalbumin (α-lac), *Xor*, *Adfp* and perilipin were analyzed by semi-quantitative RT-PCR. The graph indicates the average level of expression per gene normalized to β-actin (±s.e.m.).

alveoli (McManaman et al., 2002), butyrophilin, a hydrophobic glycoprotein found only in differentiated mammary epithelial cells (Banghart et al., 1998), the cytoplasmic, lipid-droplet-associated adipophilin protein, also known as ADFP (Heid et al., 1996), and perilipin, a marker of the adipose fraction in the mammary gland. Perilipin is normally downregulated over the course of pregnancy as the adipose fraction shrinks (Blanchette-Mackie et al., 1995). Interestingly, recently, *Adfp* has been identified as a novel hypoxia-inducible gene in MCF-7 cells (Saarikoski et al., 2002). In response to deletion of *Hif1a*, β-casein, α-lac, *Adfp* and *Xor* mRNA levels decreased by over 50% (Fig. 3B), whereas butyrophilin expression remained fairly constant (data not shown) and there was a failure to downregulate perilipin. These results indicate that there are severe deficiencies in expression of markers of milk production.

Loss of HIF1α blocks secretory activation at the transition to lactation

By day 18 of gestation, at the cusp of the transition from pregnancy to lactation, large areas of alveoli had failed to differentiate in *Hif1a*$^{-/-}$ glands, although there were areas of normal development (Fig. 4A,B). To investigate whether the areas of pathology corresponded with expression of Cre, and therefore deletion of HIF1α, Cre immunostaining was performed. As can be seen in Fig. 4C,D, even within an individual lobule, there was varying expression of Cre in the alveolar units. More importantly, the alveoli that expressed Cre were clearly those that were collapsed, and appeared to be undifferentiated. By contrast, the adjacent patches of alveoli that were negative for Cre were distended with milk precursors.

To confirm that loss of HIF1α in the mammary epithelium results in epithelial-specific downregulation of Glut1 during pregnancy, both Glut1 and Cre were sequentially detected via multiple antigen labeling on the same tissue sections (Fig. 4E-G). These studies revealed that only the Cre-negative epithelial cells expressed high levels of Glut1. By contrast, epithelial cells that expressed Cre, and, therefore, were null for

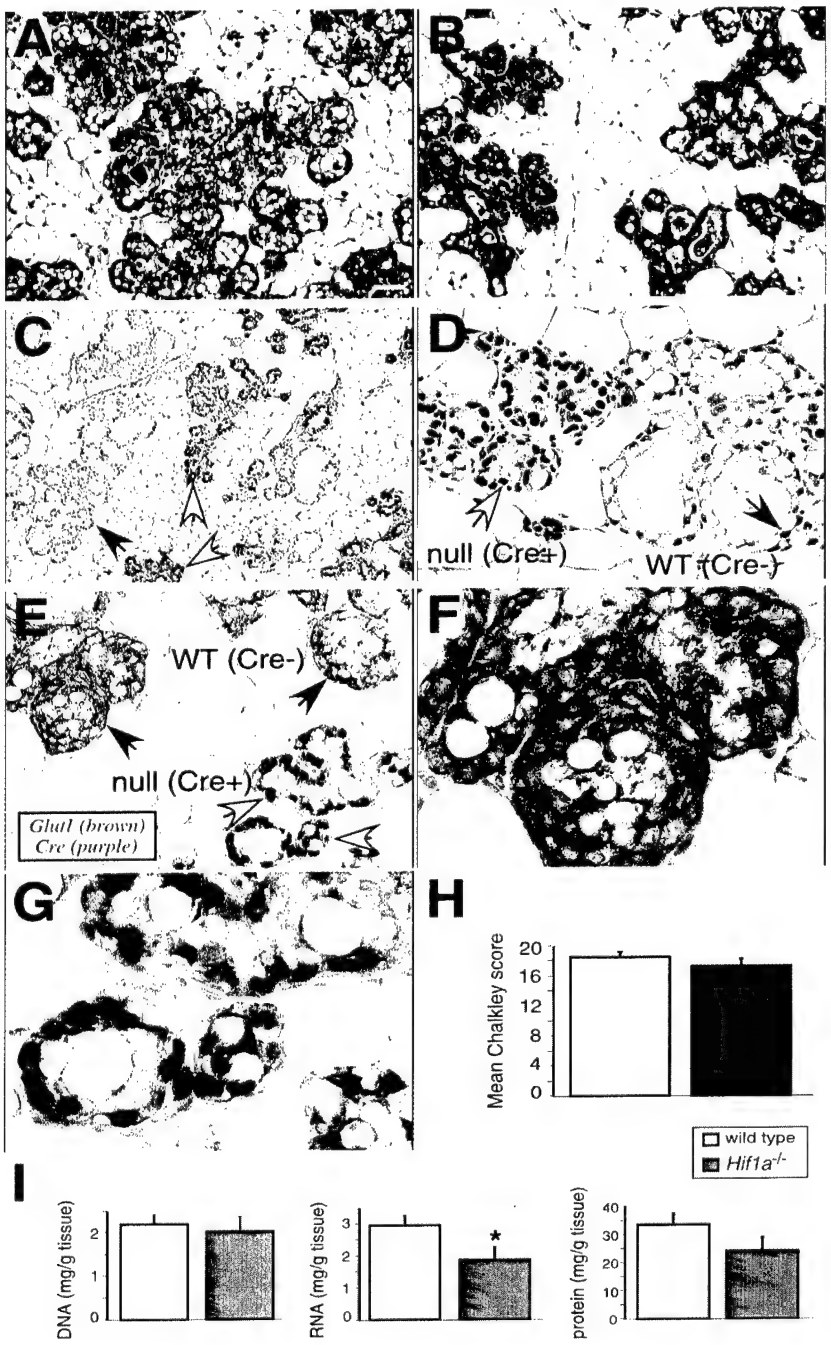

Fig. 4. Block in secretory activation at the transition to lactation. (A,B) Hematoxylin and Eosin stained sections at day 18 of gestation. Scale bar: 50 μm. Note the uniform size of alveoli and the extent of differentiation in the wild-type glands (A), versus the mixture of collapsed, non-differentiated and differentiated alveoli in the *Hif1a*$^{-/-}$ glands (B). (C) Expression of Cre (brown, nuclear staining) detected in the *Hif1a*$^{-/-}$ gland shown at higher magnification in D. Areas that expressed Cre contained small, relatively undifferentiated alveoli (null; white arrows), whereas areas negative for Cre achieved differentiation (WT; black arrows). (E-G) Multiple antigen labeling immunostaining was performed to sequentially detect Glut1 (brown stain) and Cre (purple stain, nuclear localization) using paraffin wax-embedded sections at day 18 of gestation followed by counterstaining with Methyl Green. The black arrows indicate the robust, uniform Glut1 staining pattern observed in wild-type, differentiated alveoli. The white arrows note the relatively weak Glut1 expression detected in alveoli that also express Cre (*Hif1a*$^{-/-}$). Wild-type (F) and *Hif1a* null alveoli (G) were individually imaged at higher power to highlight the reduced expression of Glut1 in response to deletion of *Hif1a*. (H) Average Chalkley score following anti-CD31 immunostaining to determine MVD at day 18 of gestation, (mean±s.e.m.). (I) DNA, RNA, and protein content per gram tissue at day 18 of gestation (mean±s.e.m.).

HIF1α, expressed less Glut1. Furthermore, compared with the uniform expression of Glut1 in individual wild-type epithelial cells, expression of Glut1 was patchy in *Hif1a*$^{-/-}$ cells. These results verify that the loss of HIF1α in the epithelium results in downregulation of direct HIF1 targets.

Because mammary epithelium-associated angiogenesis is complete by the end of pregnancy, we next analyzed microvessel density (MVD) in these tissues. Vessels were visualized by immunostaining with anti-CD31 antibodies, and the density of the vessels calculated by Chalkley counting as described previously (Ryan et al., 2000). As expected, based on the previous results of lectin staining at day 15 of gestation, there was no significant difference between genotypes in MVD at day 18 of gestation (Fig. 4H).

Finally, although there appeared to be fewer alveoli present per field in the *Hif1a*$^{-/-}$ glands, it was possible that this was an artifact resulting from the lack of alveolar cell expansion associated with differentiation. In order to compare cellularity and secretory activity, the amount of DNA, RNA and protein produced per gram of tissue was quantified. It has been previously demonstrated in the mammary gland that DNA content per gram of tissue correlates with cellularity (Knight and Peaker, 1982). As can be seen in Fig. 4I, there was no significant difference in DNA content between genotypes of mammary tissue, suggesting that epithelial cell number is equivalent at this stage of development. In support of this finding, no significant difference in the rate of proliferation of epithelial cells was observed at day 18 of pregnancy (data not shown). However, there was a significant decrease in the amount of RNA produced by *Hif1a*$^{-/-}$ glands. Finally, there was trend for decreased production of protein in *Hif1a* null glands, although because of animal-to-animal variability, this difference did not reach statistical significance.

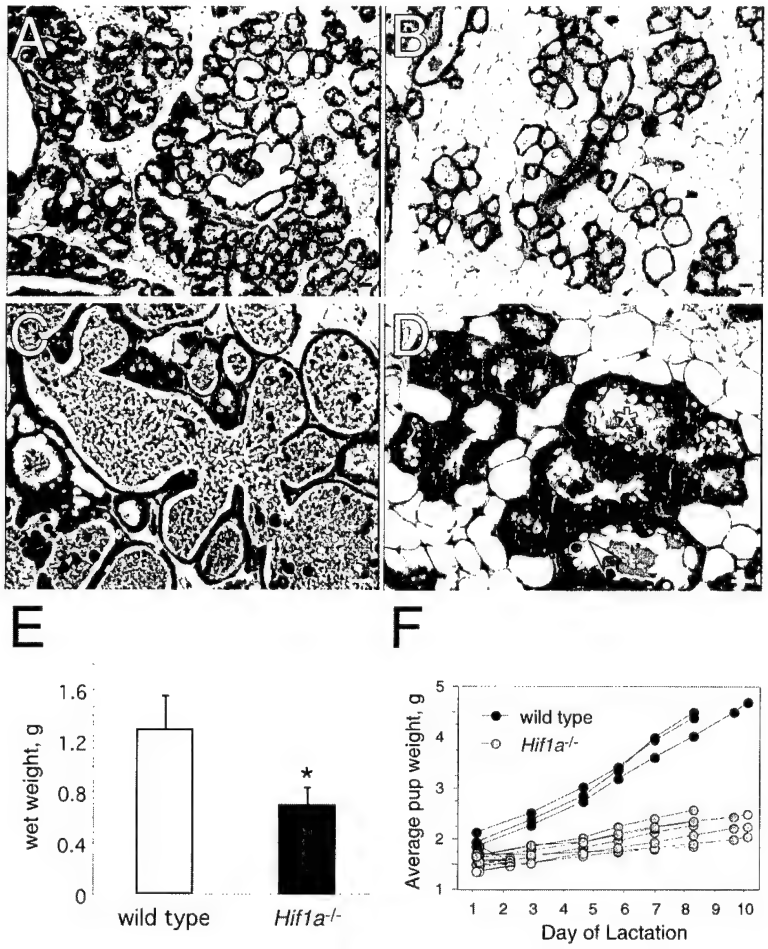

Fig. 5. Impaired secretory function at lactation. (A,B) Paraffin wax-embedded sections from mammary glands harvested on the date of birth were stained with Hematoxylin and Eosin. Scale bar: 50 μm. In comparison with wild-type (A), glands lacking *Hif1a* (B) contained fewer alveoli, which were less differentiated. (C,D) Glands were also harvested at mid-lactation from weaned dams, allowing milk (indicated by yellow stars) to fill the gland. Scale bar: 50 μm). In wild-type mice (C), the accumulation of milk fully distended the alveoli, and a relatively small volume of adipose tissue was present. By contrast, in the *Hif1a*$^{-/-}$ glands (D), accumulation of milk was minimal, large lipid droplets remained trapped within the epithelial cells (white arrow), and large areas of adipose tissue were visible. (E) Wet weight (±s.e.m.) of frozen inguinal glands harvested from lactating dams. (F) Representative growth curve of litters nursing wild-type (black circles) and *Hif1a*$^{-/-}$ dams (gray circles). Pronounced defects in pup weight gain were observed by day 3 of lactation that persisted until mid-lactation.

HIF1α is required for production and secretion of milk during lactation

The histology of mammary glands on the date of birth (day 1 of lactation) was compared without prior weaning of the litter. The *Hif1a*$^{-/-}$ glands exhibited fewer alveoli, which contained fewer milk granules, and increased evidence of trapping of lipid droplets within the epithelial cells (Fig. 5A,B). In contrast to day 18 of gestation, when expression of Cre was non-uniform, almost 100% of the epithelial cells expressed Cre recombinase by the onset of lactation (data not shown).

Glands were then compared at mid-lactation, a period of copious milk production, after a period of weaning to allow milk to fill the gland. Several defects were apparent in *Hif1a*$^{-/-}$ glands. First, in stark contrast to the wild type glands (Fig. 5C) that contained large, well-developed, expanded alveoli, large areas of fat were present in the *Hif1a*$^{-/-}$ glands, and the alveolar lumens were small (Fig. 5D). Secretory failure was also evident by the shape of the alveoli. In the *Hif1a*$^{-/-}$ glands, individual epithelial cells containing large nuclei and scant cytoplasm were still distinguishable. By contrast, in wild-type mice, the pressure of accumulated milk resulted in engorged alveoli evident by the flattened appearance of the epithelium in which the nucleus of each cell is no longer visible. Finally, although fat is normally secreted into milk as small milk fat globules, the alveoli in *Hif1a*$^{-/-}$ glands contained abnormally large droplets of trapped lipid within the epithelial cells. The reduced numbers of alveoli as well as the decrease in retained milk explain the ~50% decrease in wet weight of the *Hif1a*$^{-/-}$ inguinal mammary glands collected at mid-lactation (Fig. 5E).

Together, these defects resulted in reduced pup growth and viability. Although the pups contained milk in their stomachs, confirming normal suckling behavior, all of the pups were

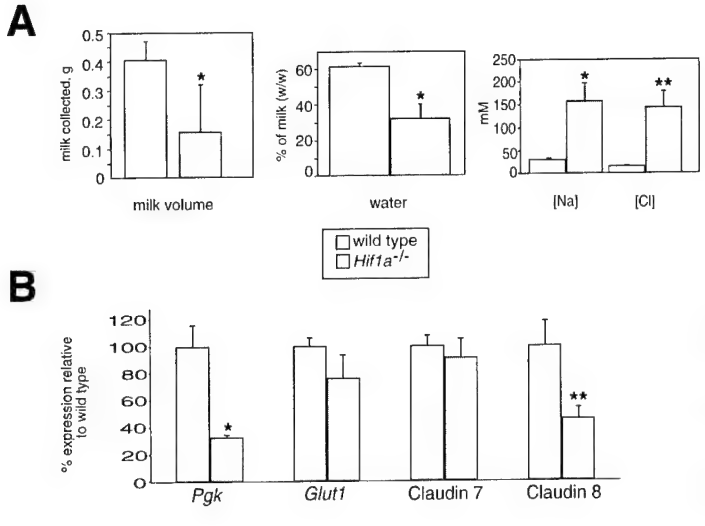

Fig. 6. Changes in milk nutrition and gene expression at mid-lactation. (A) Analysis of milk volume, water and ion content in milk collected from wild-type (white bars) and *Hif1a*$^{-/-}$ glands (gray bars) at mid-lactation, mean±s.e.m. (B) RTD-PCR was used to determine the levels of *Pgk*, *Glut1*, claudin 7 and claudin 8 mRNA in mid-lactation tissue, relative to *Ck19* expression.

runted compared with wild-type controls, and a majority died within 15 days of birth. In order to more fully characterize the differences in pup growth, pups were weighed every day after birth. As evident in Fig. 5F, the differences in weight were observed as early as day 3 of lactation, and were maintained as lactation progressed. The decrease in growth could be reversed if litters that began nursing from *Hif1a*$^{-/-}$ glands were fostered to a wild-type dam instead (data not shown), showing that the failure of the pups to grow resided in defects in the mother. Furthermore, to control for potential deleterious effects of Cre expression upon mammary gland development, lactating mice that expressed only the MMTV-Cre transgene were also analyzed. No defects in pup weight gain or mammary gland histology were noted in these dams (n=4), confirming that expression of Cre alone does not impair mammary gland development (data not shown).

Milk volume is reduced and milk composition is altered as a result of deletion of HIF1α

To determine if milk quality was affected by deletion of *Hif1a*, milk was collected from mid-lactation dams and analyzed for percentage of nitrogen, fat, water and lactose, as well as sodium and chloride ion concentrations. Several trends were noted in collection of milk from *Hif1a*$^{-/-}$ glands. First, the milk was more difficult to collect, was more viscous and was more difficult to dissolve into the water at collection, suggesting a high fat content. Furthermore, less total volume could be collected from the *Hif1a*$^{-/-}$ glands (Fig. 6A). No statistical differences were observed in protein content, whereas the amount of lactose and fat varied widely (data not shown). However, highly significant differences in water content and ion content were observed (Fig. 6A). Water content was decreased and the [Na^+] and [Cl^-] were greatly elevated in milk from *Hif1a*$^{-/-}$ glands relative to milk of wild-type glands. These changes reflected an ionic concentration closer to that observed in plasma, and indicated a fundamental failure to regulate mammary secretion properly.

Changes in gene expression at mid-lactation

Expression of HIF1 targets was also analyzed at mid-lactation following normalization to *Ck19*. In contrast to mid-pregnancy, *Pgk* expression was downregulated by 67% in mid-lactation *Hif1a*$^{-/-}$ glands, but there were no significant changes in *Glut1* expression (Fig. 6B). The decreased water content and increased [Na^+] and [Cl^-] observed in mid-lactation milk collected from *Hif1a*$^{-/-}$ dams are hallmarks of tight junction closure failure (Stelwagen et al., 1999). The family of claudin proteins is implicated in regulation of TJ strand composition (Furuse et al., 2001; Morita et al., 1999); therefore, the expression of mammary epithelial cell-specific claudin proteins was compared in mid-lactation mice. Claudin 7 and claudin 8 were specifically chosen because, relative to epithelial cell content, claudin 8 expression increases as the mammary gland progresses from mid-pregnancy to functional lactation, whereas the expression of claudin 7 remains constant (B. Blackman and M. C. Neville, unpublished observations). As expected, claudin 7 expression remained constant between wild type and *Hif1a* null glands (Fig. 6C); however, expression of claudin 8 decreased by 60%.

A requirement for HIF1α in the mammary epithelium

To confirm that the defects observed in *Hif1a* null glands were epithelial cell-autonomous, primary *Hif1a*$^{f+/f+}$ MEC were infected with an adenoviral vector expressing either β-galactosidase (control, wild type MEC) or Cre recombinase (generating *Hifa*$^{-/-}$ MEC). Infection with Adeno-Cre induced deletion of *Hif1a* in over 99% of cells (data not shown). The wild-type and *Hifa*$^{-/-}$ MEC were then transplanted into the right and left cleared, inguinal fat pads, respectively, of 3-week-old female immunocompromised *Rag1*$^{-/-}$ host mice, using a technique previously described by Rijnkels and Rosen (Rijnkels and Rosen, 2001). After outgrowth of the transplanted cells into the *Hif1a* wild-type stroma for a period of 12 weeks, the hosts were then mated and the outgrowths harvested from hosts on the date of birth of pups. Transplanted wild-type cells successfully differentiated and secreted milk (Fig. 7A, purple granules). However, the *Hif1a*$^{-/-}$ outgrowths contained small, poorly differentiated alveoli with collapsed lumens, and retained lipid droplets in the cytoplasm (Fig. 7B). In addition, the alveoli were surrounded by increased connective tissue (stained blue). Therefore, the histology of the *Hif1a*$^{-/-}$ alveoli, which regenerated in the presence of *Hif1a* wild-type stroma, recapitulated the phenotypes observed in intact *Hif1a*-null glands, confirming that HIF1α acts in an epithelial cell-autonomous manner to control mammary gland development. Glut1 immunostaining was also performed on paraffin wax-embedded sections from the transplanted outgrowths. As shown in Fig. 7C, expression of Glut1 was uniformly detected in the wild type alveoli. By contrast, in the *Hif1a* null alveoli (Fig. 7D), expression of Glut1 was decreased and patchy. Therefore, the defects in mammary gland development and physiology resulting from deletion of *Hif1a*

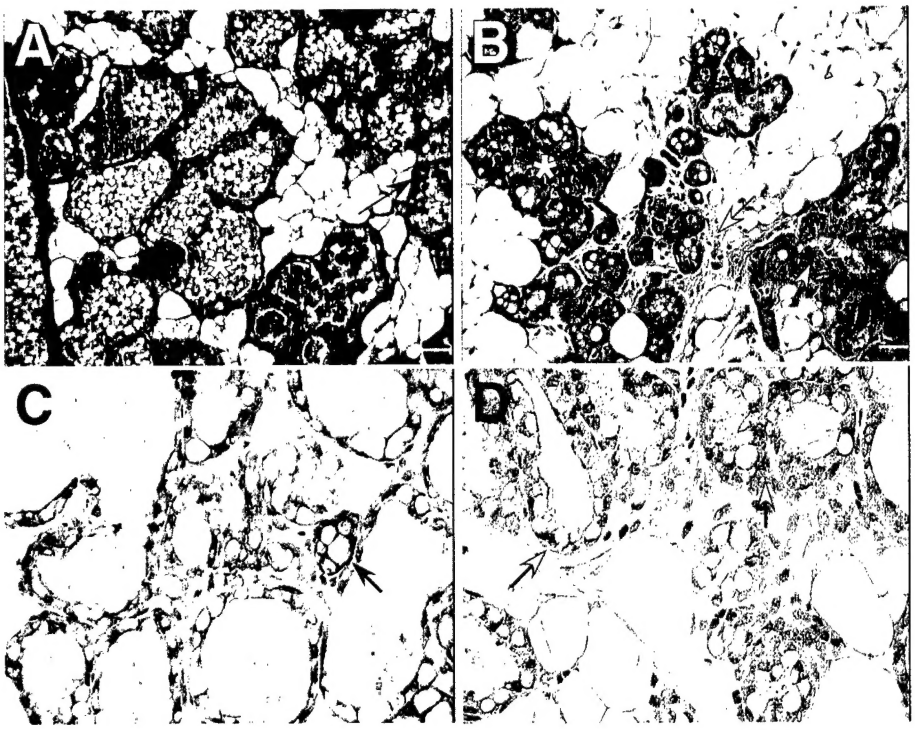

Fig. 7. The effects of deletion of HIF1α are mammary epithelial cell autonomous. Primary *Hif1a*$^{f+/f+}$ mammary epithelial cells were infected with either Adeno-βgal or Adeno-Cre and transplanted into the cleared fat pads of female host mice in order to generate wild-type (A) and *Hif1a*$^{-/-}$ (B) epithelial outgrowths, respectively. Paraffin wax-embedded sections harvested from mice on the date of birth (without prior weaning of pups) were stained with Mason's Trichrome. Scale bar: 50 μm (n=3 mice with 100% outgrowths/genotype). Outgrowths derived from *Hif1a*$^{-/-}$ mammary epithelial cells recapitulated the phenotype observed in intact *Hif1a*$^{-/-}$ glands. Note the lack of milk products (indicated by yellow stars) in the *Hif1a*$^{-/-}$ outgrowth, and the presence of large, trapped lipid droplets within the epithelial cells (B, right white arrow). In addition, there was an abnormal thickening of collagen fibers (stained blue; B, left white arrow) around the alveoli in these outgrowths compared with wild type (black arrow). (C,D) Glut1 (brown staining) was detected in paraffin wax-embedded sections prepared from transplanted outgrowths. Compared to wild type (C), expression of Glut1 in the *Hif1a* null outgrowths (D) was less intense and more patchy.

via MMTV-Cre were not due to defects in the stroma or deletion in other tissues.

DISCUSSION

We have demonstrated a requirement for HIF1α-mediated transcription in the mammary epithelium in order to produce and to secrete milk. These results indicate a novel role for HIF1α in the control of the critical transition from secretory differentiation to secretory activation and of the composition and secretion of milk at lactation. In contrast to our expectations, no changes in microvessel patterning, density or VEGF expression were noted in vivo in response to *Hif1a* deletion, demonstrating that the angiogenesis that occurs in the mammary gland during pregnancy is HIF1α-independent.

Deletion of *Hif1a* did not impact ductal morphogenesis in nulliparous mice or the proliferation of alveoli during pregnancy. Instead, defects in differentiation were observed by histology beginning at day 15 of gestation. Loss of HIF1α inhibited the expression of markers critical to secretory function, including several milk protein and milk fat globule markers. For example, expression of *Xor* mRNA was dramatically reduced at day 15 of gestation in *Hif1a*$^{-/-}$ glands. This result is intriguing in light of a recent report that mice heterozygous for *Xor* fail to properly secrete lipid into milk at lactation, ultimately resulting in pup death (Vorbach et al., 2002), a phenotype similar to that observed in response to deletion of HIF1α. In addition, expression of *Adfp* mRNA, which has recently been shown to be induced up to 70-fold by hypoxia in MCF-7 cells (Saarikoski et al., 2002), was also reduced by 50% in glands null for *Hif1a*.

Furthermore, as observed at day 18 of gestation, the failure of the alveoli to differentiate and to produce milk components corresponded completely with the expression pattern of Cre. Hence, the pronounced block in differentiation is due to loss of *Hif1a*. Consistent with this finding, transcriptional activity, as measured by RNA content per gram of tissue, was reduced by 50%. Because of the tight association observed between the presence or absence of *Hif1a* and the production of milk components in preparation for lactation, HIF1α is a critical regulator of the process of secretory differentiation in the mammary gland.

In addition, it is important to note that mammary gland DNA content was similar between genotypes at the end of pregnancy, and that no differences in rates of epithelial cell proliferation were noted at either day 15 or day 18 of gestation (data not shown). Therefore, the observed differences in histology of pregnant mice must have resulted from a failure to accumulate milk products in preparation for lactation. Of note, this mouse model is the first to date to describe defects in differentiation during pregnancy without accompanying changes in mammary epithelial cell proliferation.

With respect to loss of HIF1 function, the selective decrease of Glut1 mRNA expression by 60% at day 15 of gestation, as well as the decreased levels of Glut1 protein noted at day 18 of gestation, may explain both the defects in differentiation and lipid metabolism observed during pregnancy in *Hif1a*$^{-/-}$ mice. Normally, Glut1, the exclusive glucose transporter used in the mammary epithelium at lactation, is considerably upregulated during secretory differentiation in order to increase glucose availability (Camps et al., 1994). This is crucial because glucose is a required substrate for the production of lactose, the primary carbohydrate in milk. Furthermore, glucose transport has been proposed to be a rate-limiting factor in glucose use in the mammary gland (Threadgold and Kuhn,

1984). And, in rodents and other animals lacking the acetate-based fatty acid synthetic pathway, glucose is also used for the production of fatty acid precursors. Therefore, loss of HIF1α function during pregnancy may deprive the gland of the glucose it needs to differentiate.

The severity of the defects observed during pregnancy was demonstrated during lactation since dams lacking HIF1α in the mammary epithelium were unable to support nursing pups. Normally, secretory activity peaks at mid-lactation, but, as evident by histology, the HIF1α null alveoli contained relatively few milk granules, and large lipid droplets that are normally secreted into milk as micro-droplets remained trapped within the epithelial cells. As a consequence of defective secretion, the glands yielded less milk volume and milk nutrition was poor. In addition, the sodium and chloride content of milk was elevated, resembling concentrations observed in plasma. These changes are hallmarks of mastitis, in which the normally closed tight junctions become permeable (Nguyen and Neville, 1998). As the claudin proteins are implicated in tight junction strand regulation (Furuse et al., 2002), and claudin 8 expression was downregulated by 50%, HIF1 may also play a role in tight junction closure.

Although it is possible that the severe block in differentiation observed in the HIF1α null mammary gland prevented the transition to secretory activation, based on the previously described functions of HIF1 (Semenza, 1999), it is more likely that loss of HIF1α impaired metabolic activity at the time of highest demand: lactation. In support of this hypothesis, at mid-lactation, the normalized expression of PGK was reduced by over 67% in the HIF1α null glands.

During peak lactation, HIF1 mediation of glycolytic activity may be necessary to supplement energy production since synthesis and transport of milk components, as well as tight junction closure, are energy-dependent processes. This proposed function for HIF1 in the mammary epithelium is also supported by previous observations that increases in glycolytic enzyme activities occur at lactation (Mazurek et al., 1999). Therefore, glycolysis is likely to be necessary to maintain energy production at lactation. Interestingly, previous studies have shown that inhibitors of glucose metabolism interfere with lactation. Administration of 2-deoxyglucose, which inhibits glucose-6-phosphate metabolism in the lactating rat mammary gland, reduced lactose synthesis, as well as protein synthesis and secretion (Sasaki and Keenan, 1978). Thus, HIF1-dependent regulation of glucose metabolism may be necessary for achieving differentiation during pregnancy as well as the high metabolic rate in the mammary gland at lactation. Based on our observations of HIF1 transcriptional activity, it is possible that during pregnancy, when energy demands are lower than for lactation, successful differentiation may depend more upon Glut1 function than that of PGK. Conversely, during lactation, regulation of PGK may become more critical. The mechanisms underlying the differential impact of deletion of HIF1α on Glut1 and PGK mRNA expression during pregnancy versus mid-lactation are not clear, and will require further investigation.

In *Hif1a*$^{-/-}$ epithelial cell cultures exposed to hypoxia, VEGF mRNA decreased by ~50%. However, we were unable to detect any changes in MVD in the intact mammary gland in vivo, either by qualitative analysis of the vasculature or by Chalkley counts after CD31 immunostaining. In addition, there was no significant decrease in VEGF mRNA expression in *Hif1a*$^{-/-}$ glands at day 15 of gestation. As loss of HIF1α did not impact vasculature expansion, the mechanisms regulating angiogenesis during normal mammary gland development must be HIF1α-independent. The absence of an effect on angiogenesis in response to deletion of HIF1α is in agreement with previous results obtained by our laboratory, which showed that the MVD of both developing bone as well as fibrosarcomas remain equivalent to wild type when HIF1α is conditionally deleted (Ryan et al., 2000; Schipani et al., 2001).

Low levels of HIF1α were detectable in primary mammary epithelial cells cultured at normoxia. HIF1α stability was dramatically increased by hypoxic treatment and loss of HIF1α diminished HIF1 transcriptional activity; therefore, the hypoxic response is intact in normal mammary epithelium. Interestingly, it has been shown that HIF2α, which is structurally related to HIF1α, may also induce expression of HRE-based reporter constructs in a hypoxia-dependent manner (Wiesener et al., 1998); therefore, it is possible that HIF2α may play some role in mammary gland development. However, although both *Hif1a* and *Hif2a* transcripts are expressed throughout mammary gland development, and neither gene shows marked fluctuation in expression levels, *Hif2a* mRNA is expressed at levels approximately one-tenth of those of *Hif1a* (M.N., unpublished observations). In addition, we argue that as expression of HIF1 targets was reduced to basal levels in hypoxically stimulated primary cells lacking HIF1α, HIF2α plays little, if any, role in transcriptional regulation of HIF target genes in the mammary gland. Furthermore, because HIF2α was unable to compensate for loss of HIF1α during pregnancy and lactation in vivo, it is not likely that HIF2α is a critical mediator of mammary gland development.

Although hypoxia is the classic inducer of HIF1α stability, we have been unable to document the presence of hypoxia in *Hif1a* wild-type and null mammary tissue using reagents known to detect DNA adducts created by hypoxia, such nitroamidzole (EF5) (Evans et al., 1996). Although waves of hypoxia were readily detectable in mammary tumors, no areas of hypoxia could be detected in the normal mammary gland at any stage of development (data not shown). However, detection of adducts created by hypoxia via immunostaining may not be as sensitive in normal tissue, which can more readily adapt to hypoxia than a rapidly dividing tumor with a necrotic center.

Alternatively, hypoxia per se may not be a stimulus of HIF1α activity in the mammary gland. Several laboratories have reported that in vitro HIF1α protein is also stabilized upon treatment with insulin, insulin-like growth factor 1 (IGF1), IGF2 or activation of HER2/neu receptor upon addition of heregulin – all potent cell survival factors/mitogens for normal and breast cancer cell lines (Feldser et al., 1999; Laughner et al., 2001; Zettl et al., 1992). Additionally, a positive feedback loop between HIF1α and Igf2 transcription has been reported in human 293 cells and in mouse embryonic fibroblasts (Feldser et al., 1999). These observations are noteworthy, as IGF2 may function as a local, paracrine mitogen in developing alveoli (Wood et al., 2000). Furthermore, it has been demonstrated that the end products of glycolysis itself, pyruvate and lactate, can induce HIF1α protein stability even under aerobic conditions (Lu et al., 2002).

Recently, Le Provost et al. have deleted the partner of *Hif1a*, *Arnt*, in the mammary gland (Le Provost et al., 2002). Deletion

of *Arnt* blocked early alveolar development and impaired fertility (Le Provost et al., 2002). Based on these results, as well as transplantation of transgenic tissues into the cleared fat pads of host mice, it was argued that deletion of *Arnt* affects mammary gland development through uncharacterized, indirect effects in the ovary, although there were no differences in circulating estrogen and progesterone levels (Le Provost et al., 2002). We have not noted any differences in ovarian histology or detected recombination of the *Hif1a* locus in the ovaries of transgenic mice. Furthermore, in comparison with deletion of *Arnt*, loss of *Hif1a* impacted relatively late stages of mammary gland development. These differences are perplexing, as HIF1α partnering with ARNT is required for HIF1 activity. It is possible that *Arnt* function may be compensated for by other family members that complex with HIF1α, such as ARNT2 (Keith et al., 2001) or ARNT3 (ARNTL – Mouse Genome Informatics) (Takahata et al., 1998). Nevertheless, transplantation of $Hif1a^{-/-}$ epithelium into $Rag1^{-/-}$ female hosts revealed that the defects associated with deletion of *Hif1a* were mammary epithelial cell autonomous. Therefore, even if low, but undetectable, levels of recombination of the *Hif1a* locus were present in the ovaries in this line of transgenic mice, they have no impact on the phenotype in the mammary gland.

Because deletion of *Hif1a* specifically inhibited the synthesis of milk components during pregnancy and milk production and secretion at lactation, we argue that HIF1 activity is essential for the transition from pregnancy to functional lactation, and is also required for the maintenance of normal lactation and production of milk. Furthermore, these defects are manifested independently of regulation of angiogenesis through VEGF.

Although overexpression of HIF1α has been documented in breast tumors compared with normal tissues (Bos et al., 2001; Zhong et al., 1999), it is not clear if this contributes to tumorigenesis or is an effect of hypoxia induced by rapid proliferation. Future experiments to compare the gene expression profiles of normal mammary tissue versus mammary tumors will be useful in determining the complexity of HIF1α regulation of epithelial cell biology and secretion. As there are significant differences in how primary cells and tumor cells respond to hypoxia (Brown and Giaccia, 1998), the differential pathways that regulate these processes may prove to be excellent targets for tumor-specific, hypoxia-responsive therapeutic drugs.

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